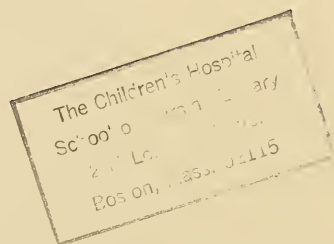


Research Report

1975/1976

Research Report
The Children's Hospital
Medical Center





**Dedicated to John F. Enders, PhD
Nobel Laureate, 1954
Chief, Virus Research Unit, Children's Hospital Medical Center
University Professor, Emeritus, Harvard University**

John Franklin Enders

On February 10, 1976, seventy-nine years after his birth in West Hartford, Connecticut, John F. Enders was the guest of honor at a surprise birthday party, attended by many of his family, former students, friends and colleagues. The occasion ended with naming the Research Building of the Children's Hospital Medical Center the *John Enders Pediatric Research Laboratories* and the unveiling of his portrait by Gardner Cox in the building's lobby. Thus, the Children's Hospital Medical Center has chosen to honor this quiet man, whose curiosity, imagination and persistent devotion to the pursuit of knowledge in the laboratory with a few close associates has brought him worldwide recognition in the form of honorary degrees and awards, including the Nobel Prize in Medicine and Physiology for 1954. The modest lettering at the building's entrance and his portrait, which looks out at those waiting for the elevator to take them to their laboratories, will provide inspiration for present and future generations of young people embarking on research careers.

What led this scion of a well-to-do Hartford banking family, who had been an oarsman at Yale, a reserve officer in the Naval Flying Corps from 1917 to 1920, and subsequently a candidate for a PhD in English at Harvard, into this extraordinarily productive scientific career, the last 29 years of which have been spent at the Children's Hospital? During this latter period, his academic rank at Harvard Medical School rose from Associate Professor of Bacteriology and Immunology, when he came to Children's, to full Professor two years after he was

awarded the Nobel Prize. Finally, in 1962, he was given the distinguished title of University Professor, and remains as University Professor, *emeritus*, in his laboratory on the thirteenth floor of the building which bears his name.

Perhaps he was destined by his genetic endowment for such a career, but it was his friendship with an Australian studying at Harvard, Hugh Ward, and his meeting with Dr. Hans Zinsser, the dynamic head of the Department of Bacteriology and Immunology at the Harvard Medical School, which diverted him from the humanities to microbiology. Zinsser, an attractive man, a scientific innovator with a strong romantic streak, whose days often began with an early morning horseback ride, was an avid investigator and stimulating teacher throughout the day, but often spent his evenings in the company of literary friends. This breadth of interest struck a responsive chord in John Enders who, although he was nearing completion of his thesis work, switched his graduate field to bacteriology and immunology and, upon receiving his PhD in 1930, became a member of Zinsser's department.

There, he and Hugh Ward carried out a series of classical experiments which demonstrated the essential role of complement as well as specific antibody in the opsonization of virulent pneumococci for phagocytosis. In his first association with the Children's Hospital, he collaborated with Dr. James Wilson, Chief Resident in Pediatrics, in an attempt to take advantage of the heat sensitivity of certain strains of pneumococci by inducing high fever in the treatment of chil-

dren with pneumococcal meningitis.

In the late 1930's, his interest turned increasingly to viruses, the basic biological properties of which had become a frequent subject of discussion with Dr. Zinsser around the lunch table in the Department of Bacteriology and Immunology. In an attempt, with William McD. Hammon, to isolate the measles virus in kittens, a new agent, the virus of feline panleukopenia, was discovered. This was followed by isolation of the mumps virus in monkeys, which led to a series of studies of the pathogenesis of the disease, and of immunity to it, which were carried out during the second World War, while Dr. Enders was a member of the Commissions on Measles and Mumps of the Army Epidemiological Board. At the same time, he worked closely with Prof. Edwin J. Cohn at Harvard in the Plasma Fractionation Program, taking responsibility for study of the spectrum of antibodies in various preparations of human gamma globulin.

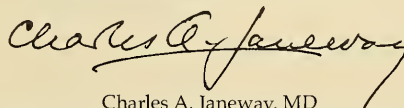
Following the war, it was a fortunate coincidence that, just when Dr. Enders was considering a move, Dr. Sidney Farber was organizing the Division of Laboratories and Research as one of the first steps in the creation of the Children's Medical Center. In this Division, Dr. Farber planned to attract a core of basic scientists, each pursuing his own research, whose presence would strengthen investigation by all the clinical departments of the hospital. John Enders was a natural choice for one of these positions. Thus, in 1947, he joined the full-time staff of the Hospital as Chief of the Research Division of Infectious Diseases.

In a few rooms across Blackfan Street from the Bader Building on the second floor of the old Carnegie Building, which had been purchased by the Children's Medical Center to provide additional research space, Dr. Enders established a quiet outpost of modern microbiology. Although he seldom left his laboratory, it had a major impact upon the care of patients with infectious disease in the hospital through the fellows, mostly from clinical backgrounds, who worked with him. Each year, this laboratory sent forth one or two of this group to fill major academic posts here and abroad, and from it issued a small but steady stream of beautifully written (thanks to Dr. Enders' background in English) scientific papers which have probably done more for the health of children throughout the world than anything else which has

emanated from this institution. It was the cultivation of the viruses of poliomyelitis and measles in the test tube, thus making vaccine development possible, that brought fame and the Nobel Prize to Drs. Enders, Robbins and Weller. However, their systematic development of simple methods for the cultivation of viruses in tissue culture and the recognition of their effects upon the cells, which could be neutralized by specific antibody, were fundamental achievements of enormous importance to public health and medicine. For these developments provided the tools for clinical and experimental studies of viral diseases just as the methods for the cultivation of bacteria and detection of antibodies developed by Pasteur and others during the latter part of the 19th century had done for the understanding, treatment and preven-

tion of bacterial infections.

By his quiet presence, by his willingness and eagerness to help young research workers through mutual discussions and sharing of experimental results, by his scientific integrity and by his persistent dedication to scholarly work, John Enders has set an example for all those who wish to pursue a career in research. We have been blessed by his presence at the Children's Hospital Medical Center. We hope we can live up to the example he has set for us, and look forward to his continuing association with the laboratories which proudly bear his name. It thus seems particularly fitting to dedicate this first comprehensive research report of the Children's Hospital Medical Center to John Franklin Enders.



Charles A. Janeway, MD
Thomas Morgan Rotch Professor of Pediatrics. *Emeritus.*

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Report of the Research Committee

Children's Hospital has for many years enjoyed a reputation for excellence not only in the treatment of patients with pediatric diseases but also in the area of biomedical research. The Trustees of CHMC have long recognized the importance of laboratory and clinical studies as an adjunct to good clinical care and the most visible expression of this concern is the JOHN ENDERS PEDIATRIC RESEARCH LABORATORIES. Built in 1970, this building is the largest single structure in the world devoted entirely to laboratory studies of pediatric disorders. It is particularly appropriate that these laboratories are dedicated to a man whose own studies at CHMC have had such a profound effect on pediatric medicine.

With the tremendous growth of research programs at the hospital and the responsibilities this growth entails, three members of the Hospital's Board of Trustees formed a Trustee Research Committee. We are particularly fortunate that Dr. David Kosowsky, Mr. Charles Hood, and Mr. David Mittell have so graciously accepted to serve in this capacity. The formation of a Trustee Research Committee is recognition of the fact that the research enterprise at CHMC has reached a most critical stage and it is essential that both staff and trustees work more closely together to solve the many problems confronting the hospital as a result of its commitment to research.

The Hospital, recognizing the importance of a strong, viable research program, has invited a number of outstanding biomedical scientists to review, on a yearly basis, the various research activi-

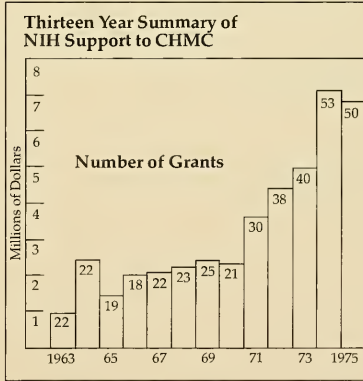
ties of staff. The first meeting of this Scientific Advisory Committee will be held on October 21, 22, and 23 of 1976. In forming this advisory body, the research committee took recognition of the fact that some method was needed to evaluate the quality of the many programs now in existence at the hospital, particularly in relationship to the changing priorities of pediatric medicine. In addition, the Hospital recognized the need for advice on what future directions research programs of the hospital should take and the types of staff required to carry them out. Thus, 1976 marks a major new thrust to effect, in an orderly manner, a review of present programs and the charting of new research directions for the future.

In 1976 CHMC reached the enviable position of becoming the fourth largest research hospital in the United States. Most of the support for our activities comes from the National Institutes of Health. With such a heavy involvement by the federal government it is inevitable that the research committee would find itself increasingly occupied with the problems of complying with the myriad rules and regulations that accompany receipt of federal funds.

In 1975 the Federal Freedom of Information Act was interpreted by the Federal District Court in Washington, D.C. to include all grants and contracts issued by the NIH. Although the research committee applauds the intent of this Act as being in the best interests of an honest and open government, it is concerned that candid dialogue between investigators at the CHMC and reviewers at the

national level may be adversely affected. In addition, there is the possibility that research designs may be pre-empted by others. Experience to date seems to indicate that the Freedom of Information Act is indeed being used to some extent for this purpose. The research committee will continue its efforts to prevent the application of the FoI Act to more sensitive documents such as privately expressed opinions and reviews of individual research proposals.

This past year also saw the application of the Federal Privacy Act to contracts awarded by the NIH. Here again, the committee appreciates the desires of the Congress to protect the privacy of citizens, but decries the application of this law to research contracts. Fortunately, the NIH has recently recognized the tremendous burden strict interpretation of this law would have on institutions such as the CHMC and has modified its regula-



tions to make it easier for us to comply with the provisions of the law. However, the committee continues to appeal that part of the regulation that would appear to require approval of the federal government for any publication resulting from contract-supported research as an unjustified infringement upon both the scientific and academic freedom of the staff.

As research has become increasingly complex, requiring the involvement of multiple institutions working together, the research committee has continued to explore ways in which very large, multi-institutional programs can be carried out without sacrificing the integrity of the CHMC or adversely affecting the conduct of research. The NIH, recognizing this problem, has developed appropriate policies for the conduct of multi-institutional programs (Consortia) and the Committee has vigorously pursued the application of these policies as being in the best interests of the Hospital, both financially and scientifically.

From a regulatory standpoint the hospital is being confronted with more and more complex regulations involving every aspect of our research activities. Regulations issued by the Occupational Safety and Health Administration, the Nuclear Regulatory Commission and the Environmental Protection Agency, and compliance with them have placed an additional financial burden upon the hospital with the result that the cost of research has now reached a point where it may be necessary to evaluate the cost-benefit of some programs. As desirable as this may be from an economic standpoint, and as desirable as the regulations may be, the committee is concerned that unreasonable application of current federal and state regulations may have a deleterious effect on the hospital's ability to properly carry out its mission as a research institution.

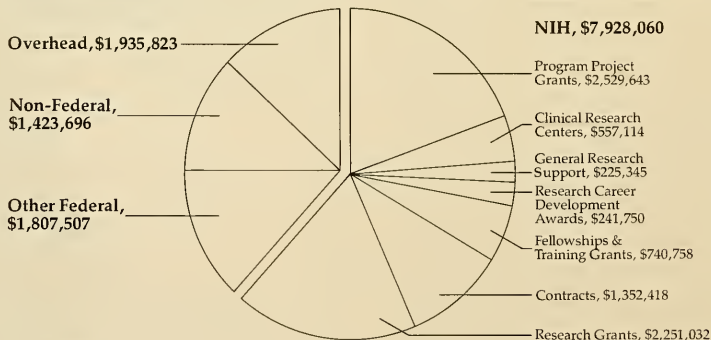
Financially, the research programs of the hospital are vigorous, although continued inflationary pressures and chang-

ing priorities at the national level continue to have an impact. Congress has begun a comprehensive review of the nation's program of bio-medical and behavioral research, and, although it cannot be predicted at this time, it is conceivable that these hearings may result in legislation that would drastically restructure the NIH. Depending upon the results of this restructuring, the hospital could face a major task in meeting whatever new demands are made upon it. The research committee, which has ultimate responsibility for approving all research programs and allocating scarce research resources, will be looking towards the Scientific Advisory Committee for counsel and advice, but we are convinced that the basic structure of research at Children's is strong and resilient enough to meet any new challenge.

Staff of the Children's Hospital are a dedicated group of concerned physicians and scientists. The quality of our programs has been recognized repeatedly by others as evidenced by the growth of our research activities over the past 10 years. Nevertheless, the continued vigor of these activities rests upon an insistence upon academic excellence and the conduct of research in an atmosphere which is conducive to the free inquiry that for centuries has been the hallmark of scientific research. To this end, the research committee, with the assistance of the Scientific Advisory Committee, is committed to maintain an appropriate environment that will retain the Children's Hospital position as a leader, not only in pediatric medicine, but pediatric clinical research as well.

Albert Broseghini, PhD
Director, Research Administration

Research Support — 1975



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Sissela Bok, PhD

Lecturer, Harvard Medical School

Marva Serotkin (Non-voting)

Director of Patient Services, Children's Hospital Medical Center

Gwen O'Sullivan

Executive Officer

Report of the Committee on Clinical Investigation

Clinical research has long been a hallmark of Children's Hospital and has always been conducted under the most rigorous ethical guidelines. However, as medicine now has increasingly sophisticated means to treat illness, the hospital has found itself confronted with moral dilemmas resulting from these increased capabilities. In order to address these dilemmas and in response to federal guidelines, the CHMC Committee on Clinical Investigation was established in 1969 to review all research programs involving humans and to provide a forum for discussion of the moral and ethical issues that might arise from such research.

Since its inception, the committee has seen an astounding increase in legislation at both the state and federal levels as the ethical considerations of research involving human subjects have been debated at all levels of society.

From October 1973 to August 1974, the Federal Government proposed 5 separate sets of regulations dealing with human experimentation. Included in these proposals were specific drafts of policies affecting particular categories of individuals such as children, pregnant women, prisoners and the mentally infirm. Since the hospital serves several of these groups, these proposals have had a major effect on the conduct of clinical research by staff and have profoundly influenced the work of the committee.

Since 1973 the Committee has included three members from outside the hospital who have brought to its deliberations a

non-medical perspective. This has proven to be extraordinarily helpful in evaluating the many complex ethical issues raised by the use of patients in research. These three members, Dr. Sisela Bok, Professor Charles Fried and the Reverend William Leach, have given much of themselves, and the hospital is indeed fortunate in having their counsel and advice.

In 1974, Congress established the National Commission for the Protection of Human Subjects of Biomedical and Behavioral Research under P.L. 93-348. This Commission has conducted a number of hearings in an attempt to formulate principles and guidelines covering research involving humans. In addition, the University of Michigan's Institute for Social Research, under contract to the Commission, has conducted a comprehensive survey of the practice of institutional review boards in the United States. The final report of the Institute will be issued in the fall of 1976, and it can be anticipated with reasonable certainty that new or modified regulations will be forthcoming as a result of this survey.

In response to the many federal and state regulations and as a result of the Committee's own experience, the Committee in 1975 codified and published its policies for the use of patients in research at Children's Hospital. This booklet has been made available to numerous institutions throughout the country and has served as source material for other com-

mittees. The hospital staff have been uniformly supportive of the Committee's efforts, and many of the current policies and procedures of the Committee were recommended by them.

In 1975 the Committee established a Consent Committee to review the use of informed consents in experimental procedures and to assist physicians in obtaining consent in difficult situations. In its first year of operation, the Consent Committee has demonstrated its value to both staff and patients, and it is expected that the functions of the Consent Committee will become increasingly important in the future.

During the past year the Committee reviewed over 125 proposals dealing with such diverse issues as confidentiality of patient records, administration of questionnaires to patients, use of new drugs and introduction of new therapeutic regimens. The steady increase in the number of protocols submitted to the Committee and the often contradictory regulations of the state and federal government have placed a great burden on both Committee members and administrative staff assigned to it. Nevertheless, the Committee and its staff will continue to carry out their mandate of protecting the rights and welfare of human subjects and furthering ethical clinical research, which is in keeping with the best traditions of the Children's Hospital Medical Center.

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Financial Support:

Genetic Aspects of the Allergic Response, NIAID, \$26,604, Dr. Colten.

Biosynthesis of Serum Complement, NIAID, \$51,344, Dr. Colten.

Effects of Carcinogens on the *In vitro* Synthesis of Complement Components, NCI, \$78,902, Dr. Colten.

Research Career Development Award, NICHD, Dr. Colten.

Dooner Laboratories, Inc., \$5,000, Dr. Colten.

Histamine Metabolism During the Allergic Response,
C.A. Hood Foundation, \$19,484, Dr. Zeiger.

Major Research Projects

I. Biosynthesis of Plasma Proteins

A. Synthesis of the Second Component of Human Complement by Monocytes in vitro.
We have recently developed a method for the preparation of cultures of confluent monolayers of human monocytes from small volumes of blood. It has been possible to maintain these pure monocyte preparations for up to sixteen to twenty weeks *in vitro* during which time the cells were capable of phagocytizing large latex beads, rosetting with erythrocytes sensitized with IgG antibody or with the third

component of complement, kill *L. monocytogenes*, and synthesized both lysozyme and the second component of complement. Evidence for C2 synthesis was based on the appearance of biologically active C2 in media harvested from monocyte cultures at timed intervals throughout the culture period. Appearance of C2 was reversibly inhibited by cycloheximide and incubation of the monocytes in medium containing ¹⁴C labeled amino acids resulted in incorporation of the label into C2 protein. Little C2 was produced during the first six days in culture. However, a marked increase in

rate of C2 production was noted after this period of time. This increase in rate of production of C2 was coincident with morphologic evidence of monocyte maturation.

B. Genetic Deficiency of the Second Component of Complement. Using the method described above direct evidence was obtained that a genetic deficiency of the second component of complement in man is due to a failure of monocytes from homozygous deficient individuals to synthesize and/or secrete C2 under conditions that support production of C2 in normal or heterozygous deficiency mon-

ocytes. This defect appears to be specific for C2 since total protein synthesis, morphology, capacity to phagocytize latex particles, and killing of *L. monocytogenes* were similar in monocytes from normal and affected subjects. The persistence of the abnormality of C2 production on prolonged culture and the observation that C2 synthesis by normal monocytes is not inhibited by C2 deficient serum indicated that this defect in specific protein synthesis is probably not the result of *in vivo* factors that might inhibit C2 production. No evidence for secretion of non-functional but antigenically identifiable C2 was obtained.

C. Genetic Deficiency of the Fourth Component of Complement (C4). Our previous investigations of a genetic deficiency of C4 in guinea pigs indicated that as in human C2 deficiency, cells normally capable of synthesizing the fourth component of complement from homozygous deficient animals failed to synthesize C4 even on prolonged culture. Fusion of these cells (peritoneal macrophages) with a cell line of human origin (Hela cells) resulted in a number of hybrid cell lines that were capable of synthesizing human C4. These data suggested and subsequent studies supported the concept that C4 deficiency in guinea pigs is the result of a lesion in the structural gene but that regulatory gene function was intact. Recently we have demonstrated cell free biosynthesis of guinea pig C4 using purified RNA from peritoneal macrophages or an S₂₀ fraction from liver homogenates as sources of messenger RNA. These methods now permit a direct search for C4 m-RNA in tissues obtained from homozygous deficient guinea pigs.

D. Genetic Deficiency of the Third Component of Complement (C3). Recent studies of a patient with a genetic deficiency of the third component of complement indi-

cated that the patient was homozygous for a blank allele at the C3 locus. Metabolic studies with purified radiolabeled C3 revealed a markedly reduced synthesis rate and only mildly elevated fractional catabolic rate. We have demonstrated, using the monocyte culture technique described above, that normal monocytes are capable of synthesizing the third component of human complement *in vitro* although no biologically active C3 was detected. Using this method, preliminary studies of monocytes from C3 deficient patients suggest that, *in vitro*, monocytes from C3 deficient individuals are capable of synthesizing C3 at rates approximately equal to those of normal individuals. Further studies to define the molecular basis of C3 deficiency are in progress.

E. Effect of Carcinogens on the Biosynthesis of Complement and Macrophage Functions. These studies were initiated following the observation that administration of nitrosodiethylamine (DEN) to guinea pigs resulted in a selective decrease in serum concentration of the fourth component of complement (C4). The decrease in C4 preceded the appearance of hepatic tumors. The biosynthesis of C2 and C4 by guinea pig macrophages was inhibited by incubating the cells in the presence of several nitroso compounds. However, DEN *in vitro* had no effect on C2 or C4 biosynthesis even at high concentrations. We considered the possibility that the effect of DEN on C4 metabolism *in vivo* was indirect. Evidence for this hypothesis was obtained in studies showing a marked decrease in C4 synthesis in cells and tissues isolated from guinea pigs fed DEN for approximately nine weeks; at that time C2 biosynthesis was unaffected. After 19 weeks of DEN administration there was an apparent depression of C2 as well as C4 biosynthesis by isolated peritoneal ma-

crophages. This seemed to be a reflection of a more generalized effect on macrophage function since decreased chemotaxis and less firm adherence of cells to glass surfaces accompanied the change in protein synthesis. Similar findings were noted with dimethylnitrosamine. In contrast, diphenylnitrosamine inhibited biosynthesis of C2 and C4 *in vitro* but had a stimulatory effect on synthesis *in vivo*. This effect seems to be "non-specific" inasmuch as a turpentine induced sterile abscess mimics the *in vivo* effect of diphenylnitrosamine. In addition to these effects of the nitroso compounds on complement biosynthesis a marked effect on opsonic activity of serum from treated animals was noted. This effect was probably independent of the complement system and was the result of a decrease in one or more of the properdin pathway proteins. Studies are presently underway to test this hypothesis.

F. Alpha 1 Antitrypsin Deficiency. In an effort to characterize the hepatic abnormality in patients with alpha 1 antitrypsin deficiency three unrelated children with the disorder (Pi types ZZ and SZ), two heterozygous and three normal subjects were studied. Histologically livers from the homozygous deficient individuals were similar to those reported in the literature. Studies of short term culture of liver tissues in the presence of radiolabeled amino acids indicated both synthesis and release of alpha 1 antitrypsin in normal control subjects and in patients with Z protein (homozygous deficient). Radiolabeled intracellular alpha 1 antitrypsin could not be found.

II. Molecular Basis of Allergic Disease

A. Cellular Immunology of Immediate Hypersensitivity. We have studied the role of human T and B lymphocytes in the pathogenesis of IgE mediated allergic reactions. T lymphocyte proliferation to

ragweed antigen E was detected in antigen E sensitive but not in antigen E nonsensitive individuals. The T cell response to antigen E was biphasic with maxima at concentrations of antigen of 20-50 microgram/ml and 10-100 nanogram/ml. Specific immunotherapy with ragweed extract resulted in a marked decrease in the T cell response at high antigen concentrations and virtually abolished the response at low antigen concentrations. Parallel studies indicated that T cells stimulated with antigen E produced a factor which, in the presence of antigen, induced B cells from antigen E sensitive donors to synthesize and secrete IgE and IgG antibodies to antigen E. B cells from ragweed sensitive individuals exposed *in vitro* to antigen E alone failed to transform or to secrete antibody to antigen E. The T cell factor had no effect on B cells of individuals not sensitive to antigen E. The results of these studies suggested that the human reaginic antibody response required T and B cell cooperation.

B. Antigen Isolation and Characterization. Sensitivity to cockroach antigens seem to be an important cause for so called "house dust" sensitivity, at least in urban populations. We have recently fractionated crude extracts of cockroach into at least three distinct antigenic species. The most important of these designated CrI is a glycoprotein of molecular weight of ap-

proximately 25,000 which is stable on boiling at neutral pH for four hours but is destroyed under mild hydrolyzing conditions in the presence of 4N acetic acid. The antigens purified from cockroach extracts are now being used for studies of T and B cell responsiveness in sensitized individuals, for provocative challenge studies in patients with asthma due to house dust sensitivity, and in immunotherapeutic trials for the treatment of allergic disease.

C. Enzymology of Allergic Disease. Thin layer radiochromatographic methods for the detection and quantitation of histidine decarboxylase and histaminase have been developed. With the use of these methods, it has been demonstrated that histaminase is specifically localized in the granule rich fraction of human eosinophils and neutrophils and not contained in monocytes, lymphocytes, or platelets.

In contrast, histamine methyl transferase is contained solely in human monocytes and is not in the other cell types examined. The physiologic role of histaminase in modulating allergic reactions was suggested by studies indicating that a variety of stimuli including phagocytosis, pinocytosis, calcium ionophores, etc., stimulate the release of histaminase from human leukocyte preparations. Release proceeds in a dose dependent fashion and is modulated by a number of factors including cyclic nucleotides and

agents that interfere with microtubular function. Preliminary data suggests that eosinophil histaminase may be induced in patients infested with a variety of parasites. Enzyme levels returned to normal following treatment of parasitic disease.

D. Hypersensitivity to Organic Dust Inhalation. Recently we have shown using clinical, immunologic and physiologic parameters that hog trypsin dust accounted for several cases of occupational asthma in a plastics plant in eastern Massachusetts. The mechanism of this respiratory disease appeared to be due to IgE mediated hypersensitivity based on direct skin testing with the putative antigen, passive transfer of IgE antibodies, antigen mediated histamine release from peripheral blood leukocytes and immediate airway obstruction in response to inhalation challenge. These effects were induced by inactivated trypsin and were therefore independent of tryptic enzymatic activity. An extension of these studies to define the role of sensitivity to pancreatic enzymes in parents of patients with cystic fibrosis have been undertaken. Trypsin is also an important antigen in production of IgE mediated hypersensitivity reactions in the parents of patients receiving pancreatic enzymes for treatment of cystic fibrosis. The basis for the infrequent appearance of such hypersensitivity reactions in patients with cystic fibrosis is under investigation.

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Financial Support:

Research into Causes and Cure of Cystic Fibrosis,
William Randolph Hearst Foundation, \$50,000, Dr. Shwachman.

Comparative Study of Sweat Test Methodologies, Cystic Fibrosis Foundation, \$17,205, Dr. Avery/Dr. Shwachman.

Care and Teaching in a Cystic Fibrosis Center, Cystic Fibrosis Foundation,
\$34,000, Dr. Shwachman/Dr. Avery.

Major Research Projects

I. Identification of the Heterozygote for Cystic Fibrosis

In this study we have employed a test based on sodium transport in the presence of glucose across the rat intestinal membrane using the Ussing chamber and measuring short circuit current at 5 minute intervals. Serum from homozygotes and heterozygotes inhibit sodium transport under the experimental conditions whereas healthy controls do not. Some other disease entities such as rheumatoid arthritis and ulcerative colitis will also inhibit sodium transport in our setup thus making the test a non-specific one for cystic fibrosis and in this regard similar to the majority of other tests which have been advocated for the detection of the heterozygote. Today we can report on approximately 40 patients with cystic fibrosis and an equal number of heterozygotes and a smaller number of healthy controls. We included sick controls as well and a few of the sick controls give us the same results as patients with cystic fibrosis. Only two out of approximately

40 healthy controls were positive for the CF factor. Of great interest is the fact that the factor in the serum is heat-labile and it is also destroyed by freezing and thawing. If one keeps the serum at refrigerator temperature the factor will not be destroyed for at least 7 to 10 days.

II. Screening Newborns for Cystic Fibrosis Utilizing Meconium and Testing for the Presence of Albumin

We have demonstrated that babies born into cystic fibrosis families, if they had albumin present in meconium, would develop this disease. However, premature babies often give a false positive result. We also found that rarely a child would develop cystic fibrosis who had a negative test for albumin. The methods we used were chemical and immunological and were never of sufficient value to report in the literature. A new procedure that was rather simple and inexpensive was developed by the Boehringer Mannheim Co. in Germany, in which a filter paper is used as a single column chromatogram in a specimen of meconium. We therefore adopted this method and have been using this method for the past

two years on specimens provided from 11 hospitals in Massachusetts and Maine.

We have examined approximately 10,000 meconium specimens to date and have detected a number of children with cystic fibrosis who otherwise would not have been found. The Cystic Fibrosis Foundation has launched a program in this country where five separate centers have been using this test.

In addition to detecting albumin by this method we have employed a quantitative technique in which we measure the amount of albumin and have developed a number of other assays such as the disaccharidases which indicates that babies with cystic fibrosis have increased activity of their intestinal mucous cells and will actively secrete the disaccharidases whereas healthy babies will not. We also found a difference in the zinc content as well as in the water content. We have also found differences in a variety of other enzymes which are known as lysosomal enzymes. We also found that in addition to albumin there are certain globulin fractions in meconium of babies with CF which are absent in otherwise healthy

babies. We have proposed a new technique of measuring the lactase which is very simple and may act as a confirmatory test when the presence of albumin is detected.

III. Enzyme Replacement in Cystic Fibrosis

This study is designed to determine the relative effect of a new pancreatic preparation called Digestaid. This preparation will be compared with two widely accepted effective preparations namely Viokase and Cotazym. This study will include careful balance studies of selected patients with cystic fibrosis who will be hospitalized for as long as 35 days. During this period they will have carefully measured diets and the influence of the pancreatic preparation will be determined by measuring their total nitrogen and fat output. They will also have a duodenal intubation to measure their pancreatic enzyme function. This study will provide information which has long been wanting and which is not available, namely, to determine on a scientific basis, the inference that one preparation that is widely used is better than any other. This will also determine the effective dose.

IV. Portal Hypertension in Cystic Fibrosis

In collaboration with Dr. Schuster, 48 patients have been treated over the past 25 years. Sixteen patients have survived as of August 1975 and all of these have had surgery. The surgery consisted in our

early period of splenorenal shunts and more recently of portacaval shunts. Only a small number of patients (8) had jaundice and 12 patients in this series had ascities. In ten patients the serum protein level was below 3 grams %.

Another study in collaboration with Dr. Schuster relates our experience in dealing with pulmonary surgery in patients with cystic fibrosis. Again this is a procedure which could result in the extension of comfort and life in patients over the past 25 years. Three patients had localized large pulmonary abscesses and the majority of patients had localized bronchiectasis or persistent atelectasis over long periods of time.

V. Long-Term Survival of CF Patients

At present we have 95 patients above the age of 25. The first 70 patients who have reached this age as of January 1, 1975 is the subject of a detailed report. An outline of the main features of this report will be presented at the International Cystic Fibrosis Congress in Paris which will be held June 1-3, 1976. This report describes the life style as well as all the medical problems and complications which occur in older patients with cystic fibrosis. We deal with occupation, educational achievements, marriage which up to now have not been dealt with in older patients with cystic fibrosis. We feel that this is the largest group of adults with cystic fibrosis in any one clinic and of course we are proud that this represents

well over 10% of our current clinic population. It is not clear why males predominate in this study and why males should live longer than females with cystic fibrosis remains a problem. Perhaps it is due to the increased activity of males where possibly better bronchopulmonary drainage may occur. We do not know if the female sex hormones are deleterious to individuals with cystic fibrosis.

A project which is now underway includes a collaborated involvement with Dr. Denise Strieder in which we measure in a scientific way the benefits derived from short-term intensive in-hospital therapy. We have long felt that this was beneficial but during the past few years we have obtained scientific evidence of a benefit as measured by a variety of pulmonary function tests including arterial blood gases. This report will also be presented in Paris at the 8th International meeting by Dr. Strieder.

A final study which is now in progress deals with pulmonary scans both ventilatory and perfusion. This is done in collaboration with our Nuclear Medicine Department, X-ray Department and Dr. Denise Strieder's Department. It is difficult or impossible to get good pulmonary function tests in small children and we feel that this technique of scanning will provide good evidence of the early pulmonary involvement that occurs in cystic fibrosis long before the ordinary x-ray can detect any change.

Division of Clinical Pharmacology

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Financial Support:

Carcinogen Metabolism by Host Intestinal Bacteria, NCI, \$117,748, Dr. Goldman.

Metabolism of Exogenous Compounds in Germ-free Rats, Milton Fund, \$3,000, Dr. Goldman.

Intensive Drug Surveillance, FDA, \$115,688, Drs. Goldman/Mitchell.

Merck Sharp and Dohme Research Laboratories, \$5,000, Dr. Goldman.

Research Career Development Award, NICHD, Dr. Friedman.

Placental Transport of Vitamin B₁₂, NICHD, \$23,209, Dr. Friedman.

Placental Transport of Vitamin B₁₂, Milton Fund, \$4,800, Dr. Friedman.

Mechanisms of Action of Vitamin K, American Heart Association, \$10,000, Dr. Friedman.

Major Research Projects

I. Effect of Intestinal Microflora on the Metabolism of Drugs and Other Exogenous Compounds

An attempt is being made to define which metabolic transformations occurring in the whole animal can be attributed exclusively to reactions of the intestinal microflora. For this purpose facilities have been established to isolate and cultivate the anaerobic flora of the intestine and to study the metabolism of compounds in isolated cultures of these organisms. Facilities have also been established to make comparative metabolic studies in germfree and conventional rats. As a result of this research it has been shown that the metabolism of the drug sulfasalazine is initiated by the intestinal microflora resulting in the liberation of 5-aminosalicylate and sulfapyridine in the colon. Thus the intestinal microflora in this case controls the metabolism of the parent drug and

results in the release of two possibly active metabolites. It is likely that the flora, by controlling the pharmacokinetics of sulfasalazine and its possibly active metabolites, may influence the efficacy of this drug. In collaboration with Dr. Richard Grand of the Gastroenterology Unit at Children's Hospital a study has been started on the pharmacokinetics of sulfasalazine and its metabolites in pediatric patients already being treated with this drug. The metabolism of sulfasalazine is also being studied in adult volunteers both under normal conditions and also where the normal volunteer has concomitant treatment with neomycin to suppress his flora.

In additional studies, by the use of germfree and conventional rats it has been shown that nitro group reduction of the model compound p-nitrobenzoic acid is reduced to p-aminobenzoic acid in the rat as the result of the activity of its flora. The implications of this are being inves-

tigated with a number of drugs and carcinogens containing the nitro group.

An additional tool in the study of biologically active intermediates arising from nitro group reduction has been an adaptation of the methods developed for the detection of carcinogens as mutagens using salmonella tester strains. It has been possible to associate these tester strains of salmonella within the gastrointestinal tract of germfree rats. When such rats are fed carcinogens the revertant response which these mutants undergo can be detected in the feces of the rats. By utilizing this model it has been possible to detect reactive metabolic intermediates that may be responsible for drug or carcinogen toxicity in the colon of animals. Investigations are now underway to explore the utility of this model for assessing the role of the intestinal microflora in the metabolism of certain carcinogens. It has been found, for example, that the carcinogen N-hydroxy-4-acetylamino-

phenyl undergoes a different metabolism in germfree and conventional animals and that thus the flora may play a role in carcinogenesis with this compound.

Additional studies have shown that metabolites arising in the metabolism of L-dopa differ in germfree and conventional animals and that certain reactions in dopa metabolism can be attributed to the flora. Experiments will soon be started to determine whether the bioavailability of L-dopa is influenced by the intestinal microflora.

II. Placental Transport of Vitamin B-12 and Action of Vitamin K

The placental B₁₂ transport process is interesting in that a high percentage of B₁₂ administered intravenously to pregnant rats rapidly accumulates in placenta. Only after a lag of several hours does B₁₂ enter the fetal circulation. The interaction of B₁₂ with human placental cell surface membranes was studied; a specific, high affinity receptor for the complex of B₁₂ transcobalamin II (a B₁₂ carrier protein) but not for B₁₂ alone has been identified and characterized.

The recent discovery that vitamin K-dependent coagulation factors are carboxylated after translation at the γ -carbon of multiple glutamate residues led to attempts to achieve K-dependent carboxylation *in vitro*. We have isolated microsomes from K-deficient rats and, after supplementing the microsomes with either a reduced pyridine nucleotide or dithiothreitol, have found K-dependent *in vitro* carboxylation (i.e., fixing of

$^{14}\text{CO}_2$ from $\text{NaH}^{14}\text{CO}_3$ to γ -carbons of polypeptide glutamyl residues). Some characteristics of the carboxylation system have been determined. A structure-activity study in which various vitamins K were compared for their capacities to support carboxylation showed that the animal vitamins K—the menaquinones—were as much as two orders of magnitude more potent than the plant vitamin—phyloquinone.

The nature of the prothrombin produced by patients treated with the vitamin K antagonists—the coumarin anticoagulants—has also been studied. Those patients are known to have immunologically normal amounts of non-functioning "prothrombin" in their plasmas. We have found that this "prothrombin" contains a spectrum of molecules that have different numbers of dicarboxyglutamate residues.

III. Intensive Drug Surveillance of Hospitalized Children

The Pediatric Drug Surveillance ("PeDS") Program at the Children's Hospital Medical Center is a prospective epidemiologic study of the clinical effects of drugs in hospitalized children. Specially trained nurse monitors collect a variety of information on consecutively-admitted patients, including certain demographic characteristics, medication history, admission laboratory values, and detailed descriptions of all drug exposures during the period of hospitalization. In addition, details of untoward occurrences are also recorded; these may be reported either as

drug-induced "adverse reactions" or as non-drug related "events." After being subjected to a number of review procedures, the data are keypunched and transferred to computer file. Appropriate epidemiologic analyses are carried out in an effort to formulate hypotheses about drugs and the clinical outcomes of their use. The purposes of the program are as follows:

1. Describe patterns of drug use.
2. Describe known adverse effects of drugs.
3. Detect previously unknown effects of drugs.
4. Describe patient characteristics which may influence drug therapy.
5. Evaluate drugs that have not yet received approval for use in certain age groups (the so-called "orphan" drugs).
6. Evaluate the various methods used to calculate drug doses in children.
7. Establish a methodology and structure that might easily incorporate clinical trials to evaluate drugs in current use, new uses for approved drugs, and new drugs in various stages of testing.
8. Develop a comprehensive data base relating to pediatric drug therapy, for use in a wide variety of clinical pharmacologic and epidemiologic evaluations.

Wards monitored to date include two general medical wards (Divisions 27 & 37), the tumor-therapy ward (Division 28) and the newborn nursery (Division 30). Over 1300 patient records have been completed, of which over 1000 are now on computer file and available for study.

Division of Endocrinology

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Harry J. Hirsch, MD, Sherry Loo, MD, Norman P. Spack, MD

Financial Support:

The Role of Glucocorticoids and Insulin in the Enzyme Activities of Certain Target Tissues in Fetal Rabbits,
The National Foundation — March of Dimes, \$10,000, Dr. Vilee.

Biochemical Responses of Progeric and Aging Cells, The Milton Fund, \$5,000, Dr. Vilee.

The Biochemistry of Diabetic Complications, American Heart Association, \$26,000, Dr. Gabbay.

Neuropathy in Diabetes: Role of Sorbitol Pathway, NIAMDD, \$54,134, Dr. Gabbay.

Urinary Polyol Excretion in Diabetic and Galactosemic Patients,
The National Foundation — March of Dimes, \$22,939, Dr. Gabbay.

Aldose Reductase Inhibition in the Therapy of Diabetes Mellitus,
Juvenile Diabetes Foundation, \$36,000, Dr. Gabbay.

Vesicular Binesis: Role in Secretory Pathways, American Heart Association, \$17,600, Dr. Gabbay.

Familial Hyperproinsulinemia: An Autosomal Dominant Defect,
The National Foundation — March of Dimes, \$20,000, Dr. Gabbay.

Major Research Projects

I. Neuroendocrine Function in Children and Adolescents—Physiological, Pathophysiological and Pharmacological Aspects

The neuroendocrine system plays a major role in the control of normal growth and development of children and adolescents, a fact easily demonstrated by patients with functional or organic lesions affecting hypothalamic-pituitary function. Our major research interests have been (1) to develop laboratory means of evaluating the multiple hormonal functions regulated by this system; (2) to determine how the system as measured by these tests is influenced by the sex and age of

the individual and how it changes in the course of development (most obviously demonstrated by alterations in hormonal production at adolescence); and (3) to apply information obtained by (1) and (2) to the longitudinal study of children with altered neuroendocrine function receiving various hormonal replacement therapies in order to define the interrelationships of hormonal activities and to determine the pharmacological requirements for therapy at different ages and stages of development. In carrying out the objectives of research interest (3), information from the acute biochemical studies is correlated with subsequent physical growth and development of the patient, the latter serving as an excellent and sensitive bio-

assay of residual hormonal function or of therapy. From studies of patients with neuroendocrine disorders, we have gained considerable insight into the hormonal requirements for normal physical growth and development of children and adolescents.

Currently, studies are underway to compare in the same subjects the changes in serum hormone levels (growth hormone, insulin and cortisol always and in some subjects LH, FSH, prolactin and ACTH) associated with sleep and those induced by pharmacological tests (glucagon, propranolol-glucagon, tolbutamide, arginine, glucose, metyrapone, thyrotropin releasing hormone [TRH] and gonadotropin releasing hormone

{GnRH}) as a function of age, sex and stage of sexual development. Past subjects of these studies have included children and adolescent aged individuals with probable genetic alterations in stature and patterns of growth and development as well as patients with functional (emotional deprivation, anorexia nervosa) or organic (CNS tumors, idiopathic hypopituitarism, cardiovascular, gastrointestinal and hematological) causes of growth failure or accelerated development. The purpose of these investigations is to define the role of hormones in the pattern of physical growth and development observed and to examine possible mechanisms by which the changes in hormonal production are brought about.

When obvious abnormalities in neuroendocrine function have been found, the effects of specific therapies (surgery, radiotherapy, and drugs for CNS tumors, hormonal replacement therapy for deficiencies including especially the evaluation of the effect of different growth hormone and human gonadotropin preparations and of drugs such as cyproheptadine, 2-bromo- α -ergocryptine, metyrapone, aminoglutethimide, o,p DDD etc. which alter hormone production) have been evaluated both by the changes observed in acute tests and by their effect on the clinical course of the patient as assayed by the subsequent pattern of physical growth and development. From the data obtained to date, it is evident that the diversity of altered neuroendocrine function that occurs both as a result of the lesions and treatment for the lesions is great and that the degree of disturbance of function may influence significantly the subsequent response of the patient to the different hormonal replacement therapies required.

II. Hormonal Regulation of Growth,

Differentiation and Senescence

Our interest in developmental endocrinology in human beings began some years ago with studies of the biochemical development of fetal adrenals and gonads and of factors which help regulate the changes observed. More recently, these research interests have broadened somewhat to include an examination of the regulatory role of some hormones (insulin and glucocorticoids in particular) on the processes of growth, differentiation and aging of certain tissues. Currently, the following studies in this area of research are being done to examine the role of hormones on growth and differentiation of fetal liver, lung and placenta and on the synthesis of collagen by the aging fibroblast.

The high morbidity and mortality observed in infants of diabetic mothers suggests that the maternal diabetic state may be detrimental to the developing fetus. Experimental evidence suggests that a hyperinsulin state exists in such fetuses. This excess insulin may block normal induction of certain enzymes by glucocorticoids as suggested by recent studies on fetal lung maturation. A project has been begun in which the offspring of pregnant diabetic rabbits are being used as models of the infant of the diabetic mother. The effects of insulin on glucocorticoid induction of phosphocholine transferase (fetal lung) and glucose-6-phosphatase (fetal liver and placenta) and of insulin and/or glucocorticoid on glycogen content of liver and placenta will be assessed. Comparable organ culture experiments will be performed using human placentas from diabetic and non-diabetic mothers some of whom will have received dexamethasone. These studies are being done to provide insight into hormonal regulation of early development and to define the effect of maternal diabetes mellitus on

fetal development.

Previous work with skin and muscle explants from patients with progeria suggested that this disorder may serve as a model of the aging process and that time-dependent changes in collagen synthesis and insulin sensitivity may represent alterations of function associated with the "aging" process. To test these hypotheses, fibroblasts from skin of human volunteers of all ages and from skin of patients with progeria have been cultured and the changes in collagen synthesis, ascorbic acid sensitivity and insulin responsiveness as a function of *in vivo* (chronologic age of patient) and *in vitro* (number of cell passages in culture) age is being assessed. Incorporation of tritiated thymidine into DNA and incorporation of ^{14}C -labeled amino acids into soluble and insoluble collagen are measured in fibroblasts at confluency. The influence of ascorbic acid and/or insulin introduced into the medium on these biochemical parameters is being determined.

III. The Biochemistry of Diabetic Complications

The Diabetes Unit of the Division of Endocrinology has a wide-ranging laboratory and clinical research program into the mechanism, development, and prevention of the long-term diabetic complications. At the core of our research program is the role and involvement of the aldose reductase-sorbitol dehydrogenase pathway (sorbitol pathway) in the development of the various diabetic complications such as cataracts, neuropathy, nephropathy and retinopathy. Basic approaches to this problem have been the purification and characterization of the aldose reductase enzyme from different tissues and species, preparation of antibodies, and the development of a radioimmunoassay system for the

quantitation and localization of aldose reductase in small amounts of tissues. *In vivo* experiments to delineate the role of the sorbitol pathway in the various diabetic complications have been performed, and the role of this pathway in the formation of cataracts and neuropathy has been most extensively studied. These experimental approaches have been aimed at determining the possible causal role of the sorbitol pathway in diabetic complications.

An additional aspect of our investigations of the sorbitol pathway is the finding that diabetic patients excrete at random large amounts of mannitol in the urine which are totally unrelated to glycosuria or the degree of diabetic control. We have demonstrated that urinary mannitol excretion is related to the ingestion of dietary fructose, and that in diabetic animals and patients, the percentage of fructose that is converted to mannitol is markedly increased when compared to the normal. The key step in the conversion of fructose to mannitol was a hitherto unknown ability of aldose reductase to metabolize fructose to a mixture of sorbitol and mannitol. The sorbitol thus formed can be recycled to fructose by the sorbitol dehydrogenase enzyme in the liver, while the mannitol cannot because of the high K_m of this enzyme for mannitol. Since the liver cell is permeable to sugar alcohols, the mannitol thus formed leaks into the serum and is cleared through the kidneys to be excreted into the urine. The fructose-mannitol conversion appears to be an insulin-sensitive step, and is accelerated by insulin deficiency. We suspect that the degree of conversion of fructose to mannitol is an index of liver insulinization.

As a practical aspect, we have been developing a number of drugs that can inhibit the aldose reductase enzyme. We

have demonstrated that an inhibitor of aldose reductase, Alrestatin, can prevent the formation of diabetic and galactosemic cataracts, and galactosemic neuropathy. We have currently an extensive program for the introduction of Alrestatin into humans, and the possible use of this drug in the prevention and therapy of various diabetic complications. We have completed Phase I study of Alrestatin in normal volunteers and diabetic patients, and those studies indicate that the compound is extremely safe without any evidence of toxicity or side-effects during the period of administration. Phase II studies involving a determination of biological activity and effectiveness of Alrestatin in diabetic patients is currently underway.

A second aspect of our investigations of the complications of diabetes mellitus has been the clarification of the biosynthetic mechanism of hemoglobin A_{1c} and other fast hemoglobin components (Hgb A_{1a} and A_{1b}) in diabetes. These hemoglobins are glycosylated *in vivo* with the glucose moiety attached to the terminal valine of the beta chains via a Schiff base which undergoes an Amadori rearrangement to form a stable ketoamine linkage. We have demonstrated that the glycosylation of hemoglobin to form hemoglobin A_{1c} occurs *in vivo* in the circulation within the red blood cells. Since the red blood cells have a 120-day life span, the measurement of hemoglobin A_{1c} and other fast hemoglobin components provides an integrated index of the blood glucose level in the diabetic patient for the prior 2 to 3 months. The serial determination of fast hemoglobin components (Hgb A_{1a}, A_{1b}, A_{1c}) will provide for the first time an accurate method for estimating the degree of blood glucose control in diabetic patients. We are currently setting up a major clinical study to evaluate the

use of fast hemoglobin component levels as a method for measuring the degree of blood glucose control, and to allow us to correlate the degree of hyperglycemia to the development of diabetic microangiopathy (as measured by quadriceps muscle capillary basement membrane thickening) and the development of macrovascular disease (as measured by evaluating the classical risk factor for atherosclerosis).

IV. Mechanism of Insulin Secretion

The study of the mechanism of insulin secretion is being conducted from a biochemical, physiological and structural approach. We are continuously trying to correlate the structure and function by these approaches utilizing both isolated islets of Langerhans and the perfused rat pancreas. Studies of the various biochemical pathways for the metabolism of glucose and correlation with structure as determined by both transmission electron microscopy and freeze fracture, have allowed us to develop new concepts for the regulation of insulin secretion by glucose. We have recently described vesicular binesis which is a process that involves membrane-membrane interaction between adjacent insulin secretory vesicles. Vesicular binesis can be quantitated by electron microscopy and we showed that it is increased 3 to 4 fold during glucose stimulated insulin release. Binesis appears to be a step in the activation of the secretory vesicles during its passage through the secretory pathway.

More recently, we have been studying the pathophysiology of nesidioblastosis, a pathological state associated with severe neonatal hypoglycemia. The pathology in this condition appears to result from a lack of maturation and/or organization of the islets of Langerhans. The islets are normally composed of at least 5 cell types (alpha, beta, delta, and 2 other

as yet undefined cell types) working together as an integrated unit. In nesidioblastosis, the islet dysgenesis appears to precipitate the severe (occasionally malignant) hypoglycemia in as yet unknown manner. The organization of islets is being studied with an immunofluorescent technique with specific antibodies to the hormones contained in each cell type, as well as by mapping the cell types by electron microscopy of individual islets. A clinical research program investigating the usefulness of somatostatin (a hormone of the delta cell) in this condition is currently underway. These studies

have led us to study and define normal islet development and organization in the perinatal period.

V. Familial Hyperproinsulinemia: An Autosomal Dominant Defect

We have recently completed a study in a kindred in whom proinsulin constituted a major fraction of circulating insulin immunoreactivity in both the fasting and stimulated states. The defect, familial hyperproinsulinemia, is inherited as an autosomal dominant defect. This defect is asymptomatic in the affected progeny with no apparent relationship to hypoglycemia or the development of diabetes

mellitus. This defect represents an abnormal species of proinsulin which is resistant to cleavage. We are currently characterizing this abnormal proinsulin and attempting to identify the point of mutation. This family is of interest because it represents the first genetic defect in proinsulin-insulin metabolism and may allow us insight into the regulation of insulin synthesis and metabolism in the beta cell, the genetics of proinsulin synthesis, and the adaptation of the organism to a primary disorder in beta cell secretory products.

Division of Gastroenterology

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Financial Support:

Bile Acid Metabolism in Infancy and Childhood, NICHD, \$45,000, Dr. Watkins.

Development and Control of Synthesis and Secretion, NIAMDD, \$35,000, Dr. Grand.

Career Development Award, NIAMDD, Dr. Grand.

Oral Treatment of Acute Diarrhea, Ross Laboratories, \$10,000, Dr. Grand.

Abnormal Caloric Supply and Wastage: Role in Severe Growth Failure in Crohn's Disease,
National Foundation for Ileitis and Colitis, Inc., \$15,000, Dr. Grand.

Follow-up Study of Neonatal Hepatitis and Liver Disease,
American Hepatic Foundation, Inc., \$2,500, Dr. Grand.

Mead Johnson Laboratories, \$1,000, Dr. Watkins.

The Center for Nutritional Research Charitable Trust, \$12,000. Dr. Watkins.

Major Research Projects

Development and Control of Intestinal and Exocrine Gland Function

The primary research interest in this laboratory is in the development, synthesis,

and intracellular processing of intestinal disaccharidases and on the development of mechanisms of synthesis and secretion of macromolecules in exocrine glands.

Particular importance is being placed on a study of intestinal sucrase. Rat intestinal

sucrase has been purified to homogeneity, and monospecific antibody raised in rabbits. Using quantitative immunoprecipitation, and solubilized sub-cellular fractions, data are being gathered regarding the kinetics of synthesis of intestinal

sucrase. In addition, the mechanism by which sucrase is transported through the cell from sites of synthesis to the brush border, the major site of its concentration in mature intestine, is being studied. Techniques involved include radioactive labeling of intestinal sucrase by the administration of amino acids orally or intravenously, preparation of sub-cellular fractions by differential centrifugation, extraction and solubilization of pure intestinal sucrase, quantitative immunoprecipitation with monospecific antibody, and measurements of intracellular radioactive precursor amino acid pools, as well as measurements of sucrase synthesis rates.

In addition, the technique of preparing sections at various levels of the villus and crypt using the cryostat have been established, and this technique is being applied to a study of sucrase synthesis under basal dietary conditions, and after high sucrose feeding.

Using fluorescent-labelled antibody, we are re-examining the developmental appearance of rat intestinal sucrase, as well as the developmental pattern of synthesis of the enzyme. The antibody-antigen system described above will also be used to study the effects of hormones on the development of the enzyme and for investigations of changes in sucrase synthesis induced by changes in dietary carbohydrate. Furthermore, the antigen-antibody system will allow elaboration of data regarding the effects of metabolic inhibitors and inhibitors of protein synthesis on the transport of newly synthesized sucrase to the brush border. Rats treated with colchicine and actinomycin will be studied.

In July 1976, we will have joining us, Dr. Aubrey Katz, a former trainee, who has had extensive experience with organ culture techniques of intestinal epithe-

lium. Using this system, human fetal intestine will be maintained in organ culture, and a variety of observations will be made. Effects of drugs (corticosteroids, thyroxine and colchicine) on morphological development, maturation of enzymes (particularly disaccharidases and depeptidases) and susceptibility to secretory toxins will be examined. Adapting studies performed in animals, synthesis of sucrase in intestine in organ culture will be studied at various stages of maturation, and under the influence of corticosteroids, thyroxine and colchicine.

We are also continuing studies in the development and control of exocrine gland function, particularly the role of cyclic nucleotides in the modulation of secretion of zymogen-contained enzymes in the parotid glands. Those studies utilize a model system established in this laboratory for the study of the effects of hormones on exocrine glands *in vitro*. Previous data showed that cholinergic and alpha-adrenergic stimulation of the rabbit parotid gland produced elevations in cyclic GMP levels and "wash-out" of amylase, while beta-adrenergic agents produced their effects on the glands through the stimulation of cyclic AMP, with a brisk secretion of zymogen-contained enzymes. The ability of adenylate cyclase in the parotid gland of the rat to respond to secretory stimuli is developmentally acquired with time during post-natal growth, and is markedly different from the pattern seen in the submandibular gland. Studies have shown that the failure of neurohumoral agents to stimulate adenylate cyclase activity in the parotid of very young animals either depends on an immaturity of adenylate cyclase or on the number of receptors for hormones in these glands. These studies are to be continued, with an effort to induce precocious maturation of the

parotid gland, using isoproterenol or corticosteroids, followed by a study of the ability of neurohumoral agents to stimulate adenylate cyclase activity and intracellular levels of cyclic AMP. Concomitantly, we will study the effects of gastrointestinal hormones and catecholamines, as well as of cholinergic stimuli, on developing pancreas and on the mature pancreas. The isolated acinar cell technique for the *in vitro* study of hormone-dependent protein release will be used. Enzyme secretion will be estimated by measuring amylase release into the medium *in vitro*, and release of total protein, and measurements of intracellular cyclic AMP and GMP will be performed.

II. Development of Bile Acid Metabolism

This work has concentrated initially on characterizing bile acid kinetics, that is the determination of bile acid pool size, fractional turnover rate of the bile acid pool, and rates of bile acid synthesis in infants and children. It has been possible to perform these investigations in small infants by utilizing deuterium labeled bile salts and a stable isotope dilution technique. This advance exploits the use of non-radioactive stable isotopes, thus eliminating the radiation hazard, which previously limited such measurements to an adult, non-pregnant population. The detection of stable, isotopically labeled substrate requires the use of sophisticated mass-spectroscopic analyses, which are being performed in collaboration with Dr. Peter Klein, at the Argonne National Laboratory. In fact, these studies represent one of several collaborations designed to apply the use of stable isotopes to investigations directed towards elucidating the pathogenesis of gastrointestinal disease.

To date, this work has progressed in three general areas: (1) investigation of bile acid kinetics as related to the devel-

opmental aspects of lipid absorption in small pre-term infants, (2) investigation of disease states characterized by abnormalities of bile acid synthesis, secretion and malabsorption, principally as the result of intraluminal phase defects, and (3) development of non-invasive, diagnostic measures of gastrointestinal function, using carbon-13 labeled substrates.

Characterization of the developmental aspects of lipid absorption, and in particular, studies concerning the regulation of bile acid kinetics, have resulted in several interesting findings. First, we have demonstrated that the full term infant and, to a greater extent, the premature infant has markedly reduced intraluminal bile acid concentrations, as compared to older children and adults. Bile acid synthesis rates were found to be increased. However, the bile acid pool size was diminished as were the intraluminal bile acid concentrations. It was found that these parameters of bile acid metabolism are profoundly influenced by medications administered to the mother before delivery and appear to relate directly to the functional maturity of the enterohepatic circulation for bile salts. This immaturity profoundly influences the capacity of the infant to solubilize, and thus absorb, the products of lipolysis. The impact of this defect is poor lipid absorption which has profound nutritional significance for the developing infant, and represents a major area for future investigation. Further studies are now in progress to elucidate the mechanisms of, and to determine the factors central to, the development of efficient lipid absorption in the neonate. These studies are investigating the influence of dietary lipid composition on intraluminal bile acid concentration, bile acid kinetics, combined with its effect on the integrity of the enterohepatic circulation for bile salts.

More recently, in order to exploit the technological advances made possible by multiple ion monitoring, with GC-mass spectroscopy in the chemical ionization mode, simultaneous determination of bile acid kinetics in plasma and in bile are being conducted using newly synthesized carboxyl labeled C-13 bile acids. These investigations will make possible investigation of bile acid kinetics without repetitive duodenal aspiration and will permit us to extend such investigations to include children with abnormalities of lipid metabolism, specifically those with hypercholesterolemia, by a non-invasive means, without radiation hazard.

Having acquired knowledge concerning the normal developmental patterns of bile acid metabolism in childhood, studies detailing specific abnormalities of bile acid metabolism in diseases with gastrointestinal and hepatic dysfunction are possible. For example, examination of bile acid kinetics in children with cystic fibrosis are being conducted in order to evaluate mechanisms responsible for the profound fecal loss of bile acid found in this disease. Characterization and identification of bile acids excreted in children with cholestatic disease, and neonatal hepatitis, or biliary atresia, are being performed in order to evaluate the hepatic excretory mechanism operant in these poorly understood syndromes.

To aid further in characterizing gastrointestinal function, other Carbon-13 labeled substrates have been specifically developed for use as breath tests. These substrates contain, in principle, a "target bond", which following the absorption and/or utilization of the substrate, results in the elaboration of C-13 CO₂ which may be detected in the expired air. Studies designed to test the methodological limitations of detection and to determine rates of endogenous C-13 CO₂ produc-

tion have been completed. Several substrates including C-13 trioctanoin and C-13 tripalmitin have been synthesized and are being utilized to detect and to investigate mechanisms of fat malabsorption in children. In addition, C-13 glycocholate is being investigated as a substrate to detect syndromes of bacterial overgrowth and to measure distal ileal function in children with persistent diarrhea or other malabsorptive syndromes.

III. Growth Failure in Inflammatory Bowel Disease

Growth failure is a common and troubling complication of ileitis and colitis in children and adolescents. The causes of this problem have not been determined, but altered function of hormones does not appear to be the primary abnormality. Rather, it may be that bowel disease increases the caloric needs of such patients to a degree which cannot be met because of poor appetite, abdominal cramps, and diarrhea.

The present studies are designed to investigate the possibility that growth failure results from increased and unsatisfied energy requirements. Energy consumption data and details of body composition will be obtained: (1) From determination of energy (caloric) intake, utilization and excretion; (2) From measurements of oxygen consumption, CO₂ production and urinary nitrogen excretion; (3) From metabolic balance studies of fat, nitrogen and electrolytes; (4) By measurement of lean body mass using ⁴⁰K counting. These data will be compared to those obtained during nutritional support therapy and at a time following this therapy in each patient. In addition, studies of endocrine function will be obtained in each patient. It is anticipated that the data will provide a basis on which to establish new methods of therapy for severe growth failure.

Division of Hematology and Oncology

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National Foundation Basil O'Connor Starter Grant, \$18,700, Dr. Alter.

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Lipid Metabolism: Plasma and Membrane Lipoproteins, NHLI, \$67,531, Dr. Lux.

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Leukocyte Oxidase Activity and Susceptibility to Infection, NIAID, \$53,555, Dr. Nathan.

DNA Polymerases in Leukemia, Research Career Development Award, NCI, Dr. McCaffrey.

Cancer Research Scholar of the Massachusetts Division of the
American Cancer Society, Dr. Scher.

Major Research Projects

I. Red Cell Studies

A. Prenatal Diagnosis. Prenatal diagnosis of sickle cell disease or thalassemia has been performed in Dr. Alter's laboratory on more than two dozen patients.

Fetal blood samples are obtained by placental aspiration or fetoscopy in London, England, or New Haven, Conn. The samples are labeled with radioactive amino acids and then shipped to Boston where their globin chain synthesis is analyzed by column chromatography. Dr.

Alter's current research is directed at finding methods to analyze samples containing a mixture of fetal and maternal blood. Since fetal red cells are larger than adult red cells, efforts are being made to separate them on the basis of density. Fetal and adult red cells also differ in-

igenically and thus may be separable by differential agglutination or hemolysis. A laser-activated cell sorter is also being employed to help separate these cells on the basis of their size and antigenicity.

Dr. Alter's group is also developing new and simpler methods of globin chain analysis using principles of column chromatography, electrophoresis, and immuno-affinity.

B. Erythroid Cells in Tissue Culture. Mouse erythroleukemia cells which grow in culture and differentiate in response to exogenous agents are being used to study hemoglobin regulation and erythroid maturation. These erythroleukemia cells are important and useful *in vitro* models of erythroid differentiation since the cells respond to dimethylsulfoxide (DMSO) with morphologic maturation and hemoglobin production. Dr. Alter's group is currently investigating agents other than DMSO which lead to different morphologic changes and which appear to stimulate the production of globin chains but not hemoglobin. Studies of the types of globin chains produced in the erythroleukemia cells when treated with the different agents are also underway since some globin chains seem to be stimulated selectively, and his selection varies with the different inducing agents.

The mouse erythroleukemia cells have been fused with human fibroblasts forming heterokaryons which survive for a few days and which produce hemoglobin. So far, the type of hemoglobin made in the fused cells appears to be coded for by the mouse rather than the human parent cell line. The cells will be manipulated, and the chemicals which successfully induce globin synthesis in the erythroleukemia cells alone will be employed in cultures of the fused cells. These efforts will be directed toward elic-

iting production of human hemoglobin by the fibroblast partner of these cell heterokaryons.

If hemoglobin synthesis can be induced to occur in fibroblasts, then prenatal diagnosis of hemoglobinopathies could be done by the use of fetal fibroblasts from amniotic fluid which are much easier to procure than fetal red blood cells.

C. Red Cell Membrane Studies.

1. Characterization of Spectrin. Spectrin is extracted from red cell ghosts and purified by gel filtration chromatography. The protein is homogeneous by several criteria and is currently undergoing extensive physical and chemical characterization. In Dr. Lux's laboratory these studies are providing important information about the composition, conformation, size, shape, state of aggregation, spectral properties, and enzymatic functions of spectrin. Additional studies are in progress to determine the nature of the interaction between spectrin and other membrane proteins, particularly red cell actin and to compare the properties of spectrin from patients with certain congenital hemolytic anemias with the normal molecule.

2. Irreversibly Sickled Cells (ISC's). ISC's are circulating red cells in patients with sickle cell disease which retain a sickled shape even when oxygenated (i.e. when their hemoglobin is not "Sickled"). Several observations indicate that the ISC is caused by a membrane defect acquired during repeated sickling. ISC's are rigid cells and have a very short life span. They make a major contribution to the hemolysis of patients with sickle cell disease and may contribute to the recurrent painful crises experienced by these patients. Dr. Lux's group has been comparing membranes of ISC's and reversibly sicklable cells (RSC's) with normal ghosts to better define this membrane defect.

Collaborative studies are in progress with Dr. Bertil Glader who is examining the permeability properties of ISC's. Dr. Lux has shown that many ISC's form ISC-shaped ghosts. When the membrane cytoskeletons of these ISC-shaped ghosts are prepared by treatment with a non-ionic detergent, they too retain the shape defect. The protein composition and electron micrographic structure of the ISC cytoskeletons are normal. These studies isolate the defect in the ISC to spectrin, actin, or one of the other minor protein components of the membrane cytoskeleton. It appears that this cytoskeleton is "fixed" in the sickled shape during deoxygenation and subsequently is unable to resume the normal biconcave form. The process(es) responsible for this fixation are currently under study.

3. ATP-Depleted Red Cells. The ATP-depleted red blood cell is a model system for study of the factors which influence red cell membrane deformability. Abundant indirect evidence suggests spectrin is involved in the control of membrane deformability, but there is little direct evidence to substantiate this premise. Reasoning that if spectrin does control red cell membrane deformability, it must exist in at least two physical states, Dr. Lux's group has been comparing properties of spectrin from fresh (deformable) and ATP-depleted (indeformable) red cells. They have found that spectrin in ATP-depleted red cells is more tightly associated with the membrane (i.e. is less "extractible") than normal, that the accumulation of inextractible spectrin closely parallels the changes in membrane permeability and deformability which characterize ATP-depleted red cells, and that the change in spectrin extractibility is reversible if ATP levels are restored to normal. The process is complex since spectrin becomes highly poly-

merized as well as inextractible during ATP depletion. The mechanisms responsible for this change in spectrin properties are currently being investigated.

4. Red Cell Membrane Phosphorylation. Spectrin is specifically phosphorylated by a cAMP-independent membrane protein kinase. The significance of this phosphorylation is unknown, but the possibility that spectrin phosphorylation may influence red cell shape or deformability has not been overlooked. Recent reports suggest that red cell membrane phosphorylation may be defective in certain hemolytic states, most notably hereditary spherocytosis (HS). However, this observation is controversial and complicated by the fact that membrane phosphorylation assays are exceptionally sensitive to slight variations in assay conditions. In order to clarify this increasingly confusing situation, Dr. Lawrence Wolfe in Dr. Lux's group examined red cell membrane phosphorylation in HS patients using a newly described membrane phosphorylation assay and developed a method for analyzing membrane protein phosphorylation in the intact red cell. These studies indicate that physiologically relevant membrane phosphorylation processes are normal in HS red cells and that the defective phosphorylation observed by others in isolated HS ghosts is a function of the assay conditions and is not the basic molecular defect in HS patients. More importantly, these studies provide a relevant method for testing phosphorylation in other hemolytic anemias.

5. Red Cell Membrane Permeability. These studies are an attempt to define the physiologic variables that influence erythrocyte membrane function, particularly those factors regulating cation permeability and cellular hydration. Several different approaches to this problem are employed. (1) Study of genetically abnormal

red cells which have abnormal cation permeability. (2) Study of the effects of various drugs on cation permeability and cell hydration. Currently the membrane permeability effects of propranolol are being investigated in several different species of erythrocytes. The different drug effects are being examined in light of the other known functional differences between these red cells. (3) Study of the relationship between hemoglobin properties and alteration in membrane permeability and cell water content. Cells containing mutant hemoglobins (such as HgbS) manifest marked changes in membrane permeability when the conformation of the hemoglobin is altered (i.e. deoxygenation of sickle red cells). Similarly the oxidative degradation of normal hemoglobin to Heinz bodies also affects the processes which regulate cellular hydration. (4) Study of the relationship between cell metabolism and membrane permeability. Genetic abnormalities of red cell metabolism (such as pyruvate kinase deficiency) frequently are associated with alterations in membrane permeability. Various *in vitro* perturbations in cell metabolism also change cation and water permeability.

D. Treatment of Acquired Hemochromatosis. The advent of high transfusion regimens as a mode of therapy for thalassemia has markedly improved the quality of life of these patients, but it has not altered their poor long-term prognosis and may actually accelerate the development of siderosis and its terminal complications. Until recently there has been no effective way to specifically remove the large amounts of tissue iron that accumulate in these patients. A specific, safe iron chelating agent, desferrioxamine, is available, but only small amounts of iron are removed when it is given in an intermittent, intramuscular dose which is rec-

ommended procedure. Dr. Propper has discovered that continuous administration of desferrioxamine is a vastly more effective way to remove iron from these patients. He has perfected a technique for continuous subcutaneous administration using a small portable pump which the patients can manage at home. Initial results with this technique are exceptionally encouraging. The method appears to be both safe and effective. A small group of patients are now being maintained in negative iron balance on a high transfusion regimen and preliminary data indicate improved cardiac performance.

II. White Cell Studies

Phagocytosis is a complex cycle of events which includes recognition by the phagocytic cell of the material to be ingested, the ingestion process, the metabolic events associated with ingestion, and the oxidative metabolism believed to be responsible for bacterial killing.

Currently Dr. Cohen is examining the effects of physiologic and pharmacologic agents on the ingestion process and the formation of active oxygen metabolites such as superoxide and hydrogen peroxide. He uses dye-coated particles to measure ingestion quantitatively, Cytochrome C to measure superoxide production spectrophotometrically, and scopoletin and horseradish peroxidase to measure hydrogen peroxide formation fluorometrically.

Dr. Cohen has begun to isolate and characterize the enzyme system in polymorphonuclear leukocytes which is responsible for superoxide generation. It has been found to be a reduced pyridine nucleotide oxidase and is found in the particulate fraction of the cell. Kinetic analysis suggests that the *in vivo* substrate is probably NADPH, but purification of the enzyme is necessary before the substrate specificity and the charac-

teristics of the enzyme can be accurately determined. Similar studies are being performed on alveolar macrophages to document the presence of this system in other phagocytic cells.

Hopefully, an understanding of the mechanism of activation of this enzyme system will expand our knowledge of how phagocytic cells protect against invasion by noxious agents. In addition, purification of the enzyme will permit investigation of this system in chronic granulomatous disease, a serious congenital disease in which bacterial killing is defective.

III. Studies of Childhood Leukemias

A. DNA Polymerase in Leukemia. Dr. McCaffrey laboratory has shown that the presence of the thymic enzyme terminal deoxynucleotidyl transferase (TdT) is a distinct biochemical property of acute lymphoblastic leukemia (ALL) cells; it has been identified in ALL cells from 35/37 patients. The absence of this enzyme in leukemic cells of patients with acute myeloblastic leukemia (AML) makes it a useful biochemical criterion for resolving morphologically equivocal cases. Further, Dr. McCaffrey has found that 10 out of 30 chronic myelogenous leukemia (CML) patients in blast crisis have TdT-positive blast cells. This suggests a relationship between such CML blast cells and ALL blast cells. A therapeutic trial is being organized to determine whether, in such CML patients, drug combinations which are effective in ALL might be of similar benefit.

He has also shown that normal human bone marrow contains a population of TdT-positive cells. The characterization of this cell population, and especially its relationship to those normal thymus cells which are TdT-positive, will be a topic of major concentration over the next year. Preliminary studies suggest that this nor-

mal marrow TdT-positive cell compartment is lymphoid in nature and has prothymocyte characteristics. One (or both) of the normal TdT-positive cell populations in thymus or bone marrow may be the target cell populations for the event(s) precipitating ALL. Therefore, the isolation, characterization, and eventual progation *in vitro* of these normal TdT-positive cells should be of enormous importance.

Subtle differences exist between leukemia-associated and normal marrow TdT. Dr. McCaffrey is attempting to develop discriminating criteria between these possibly different isoenzymes. The availability of such a technique would be extremely useful in biochemically assessing bone marrow for residual leukemic cell populations.

Dr. McCaffrey has developed a fluorescence-antibody assay system for TdT in association with Patrick Kung at the Center for Cancer Research at the Massachusetts Institute of Technology. Pilot studies are now underway. In the first group of patients studied, there has been absolute agreement between biochemically positive and negative samples and FA-positive and negative samples. The FA assay system will facilitate rapid classification of acute leukemias into TdT-positive and negative categories. It will also be useful in identifying TdT-positive cells in heterogeneous cell samples.

More recently Dr. McCaffrey has identified an apparently novel DNA polymerase in chick bursa homogenates. It shares with TdT the ability to add dNMP's randomly from dNTP substrates to 3'-OH ends of preformed DNA molecules. Unlike TdT, however, this new activity requires a double-stranded structure for activity. Purification and tissue distribution studies of this new enzyme

are underway.

B. Effect of Chemotherapeutic Agents on Macromolecular Metabolism. Dr. Abelson's studies include detailed experimentation on the effects of methotrexate on growing normal and lymphoblastic lymphoid cell cultures as well as on resting and growing 3T6 fibroblasts. Resting 3T6 fibroblasts are resistant to very high concentrations (10^{-3} molar) of methotrexate for long periods of time. When resting cells are exposed to methotrexate during and after serum stimulation which induces semi-synchronous entrance of the cells into S phase after a twelve-hour lag period, the cells continue to be resistant to the effects of high doses of methotrexate if the drug is removed prior to the onset of DNA synthesis. Resistance is measured by the ability of the treated cells to survive the effects of methotrexate and to retain clonogenicity. In addition, it is clear that actively growing cells must be exposed to the drug for at least a minimum amount of time in order for cytotoxic effects to be realized. This minimum interval for cell killing has not been determined for 3T6 cells but is in excess of two hours at a concentration of 10^{-3} molar. It should be emphasized that concentrations of methotrexate greater than 10^{-6} molar will produce an immediate cessation of the ability of the cells to incorporate deoxyuridine into DNA. Normal lymphoid cell lines are sensitive to killing by methotrexate. There is, however, a great discrepancy in the suppression of deoxyuridine incorporation as compared with the ability of the cells to continue other macromolecular processes. In addition, there seems to be cell-specific changes in nucleic acid and protein synthesis which lend various interpretations to the mode of action of methotrexate cytotoxicity. Dr. Abelson is attempting to unify these disparate and

often conflicting notions of the mechanism of action of methotrexate. The ultimate aim of these studies as well as others involving 6-MP, hydrocortisone, and certain phase I agents is the identification of specific chemotherapeutic targets which would enhance our ability to rationally design protocols for combination chemotherapy.

Additional studies are underway in Dr. Abelson's laboratory to determine whether brain tumors contain the enzyme dihydrofolate reductase. This enzyme has been previously considered to be the principal target for the drug, methotrexate. Inhibition of dihydrofolate reductase by methotrexate results in a diminished pool of reduced folate co-factors resulting in decreased purine and pyrimidine synthesis *via de novo* pathways. Recently, normal brain from a variety of mammalian sources has been reported not to contain the enzyme dihydrofolate reductase. Reduced folate co-factors must therefore be transported into the brain from the blood or cerebral spinal fluid. It has now been determined that 5-methyl tetrahydrofolate is actively pumped from the blood into the cerebrospinal fluid at the level of the choroid plexus. This is a saturable process and is inhibited by methotrexate. Dr. Abelson's laboratory has preliminary evidence from six human brain tumors of the type glioblastoma multiforme. These results indicate that there is no activity for the enzyme dihydrofolate reductase in these brain tumors. If these results are substantiated, they may have important implications for a new chemotherapeutic approach to brain tumors. A specific inhibitor of either the purine or pyrimidine salvage pathway would theoretically allow the selective treatment of brain tumors. If brain tumors are deficient in the enzyme dihydrofolate reductase and

are treated with an agent which is selectively toxic for one of the salvage pathways, then there should be a selective cell kill in the brain tumor versus the rest of the body. It should be remembered that the liver has large amounts of the enzyme dihydrofolate reductase and can therefore provide reduced folate co-factors for *de novo* pyrimidine and purine synthesis. Normal brain has little if any requirement for cell division although RNA synthesis is active. This problem will be further explored with brain tumor material as well as with glial cells in culture. This work suggests the potential for a unique approach to the treatment of cancer which exploits the inherent biochemical differences between the various cells in the body.

C. Viruses that Induce Murine Leukemia.

1. *Abelson Leukemia Virus*. Dr. Scher's group has shown that Abelson virus (A-MuLV) has transforming activity. They have developed a quantitative transformation assay for 3T3 cells. A-MuLV is defective and requires a helper virus in order to replicate. Together with Moloney leukemia virus (M-MuLV), A-MuLV causes B-cell lymphoid tumors in mice. Dr. Scher has recently shown that the A-MuLV (A-MuLV) combination, but not M-MuLV alone, can transform hematopoietic fetal liver cells. The A-MuLV-transformed lymphoid cells have the morphology of lymphoblasts, and recently Dr. Scher, in collaboration with Dr. R. Parkman (Immunology), has shown that they lack the θ antigen of murine T-cell lymphomas. Long-term cultures of these cells also lack murine surface immunoglobulins. Lymphoid tumors induced *in vivo* in mice with A-MuLV (M-MuLV) lack both the θ antigen and surface immunoglobulins. Studies of immunoglobulin synthesis in these cells are currently in progress.

2. *Moloney Sarcoma Virus*. Dr. Scher's group has been comparing A-MuLV with Moloney sarcoma virus (MoSV). Although the two viruses have many similar properties and appear to have regions of genetic homology, Dr. Scher has found distinctive differences in the types of tumors caused by these viruses in mice and in their ability to transform different cell lines.

3. *Kirsten Sarcoma Virus (KiSV) and Harvey Sarcoma Virus (HaSV)*. Dr. Scher's group has shown that these two defective viruses when combined with the M-MuLV "helper virus" cause erythroid leukemia in newborn mice. Experiments in hypertransfused mice indicate these erythroleukemic cells can replicate in the absence of erythropoietin.

D. *Tissue Invasion by Viral-Transformed 3T3 Cells*. Since malignant tumors are characterized by their ability to invade surrounding normal tissue, it is important to examine this property in transformed cells. Dr. Scher has devised a model system for studying tissue invasion using the chorioallantoic membrane of the chick embryo. 3T3 cells, transformed with KiSV or A-MuLV, are inoculated onto the chorioallantoic membrane (CAM). They invade the ectoderm of the CAM and within 2 to 5 days have produced tumors in the mesoderm of the CAM. The invasiveness can be quantitated, e.g., 5×10^5 viral-transformed cells cause tumors while 2×10^4 cells do not. These viral-transformed tumors also attract host blood vessels, a characteristic of natural malignant tumors. Additional definition of this very interesting model system is currently underway.

E. *Human Serum and Pituitary Factors that Stimulate 3T3 Cells to Divide*. Dr. Scher's group has isolated a 13,000 molecular weight polypeptide from human

serum that stimulates division in confluent populations of 3T3 cells. A similar factor has been isolated from bovine pituitary glands by others. Dr. Scher is pres-

ently investigating human pituitary glands (in collaboration with Dr. M. Rabin, New England Medical Center) to see whether it also contains this polypep-

tide and whether this is the source of the material in serum. Future studies will focus on the nature of the interaction of this factor with 3T3 cells.

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 Immunologic Mechanisms in Renal Diseases in Childhood, NIAMDD, \$150,488, Dr. Rosen.
 The Molecular Basis for Hereditary Angioneurotic Edema, NHLI, \$15,151, Dr. Rosen.
 Preparation and Clinical Trials of Intravenous Gamma Globulin, NHLI, \$18,711, Dr. Rosen.
 Tolerant Cell and Cancer Antigen, NCI, \$72,308, Dr. Borel.
 The Massachusetts Lupus Foundation, \$2,000, Dr. Borel.
 Prevention of Warm Hemolysis by the IgG Effector Site for the Monocyte Receptor,
 Charles H. Hood Foundation, \$19,000, Dr. Rosen.
 Hybrid Tumor Cells: An Immunotherapeutic Agent, American Cancer Society, Inc., \$68,642, Dr. Parkman.
 Immunologic Tolerance to DNA in Murine Lupus Nephritis, NIAID, \$47,073, Dr. Borel.

Major Research Projects

I. Genetic Defects in Complement

The pathogenesis and pathophysiologic consequences of C3, C2, C3 inactivator deficiencies are under study. Genetic polymorphisms of Factor B, C3, C2- and C6 have been described and extensive linkage studies are being carried out in relationship, particularly to the major histocompatibility locus.

II. Cellular Basis of Immunodeficiency
 Subsets of normal lymphoid populations have been described together with factors which enable cell cooperation be-

tween B and T cells. Immunodeficiency disease has been examined in light of absent or dysfunctional subsets of lymphoid populations. The biochemical and cellular consequences of adenosine deaminase deficiency is under investigation with particular regard for the consequences of this enzyme defect in the maturation of lymphoid cells.

III. Molecular Basis of Immunodeficiency

The IgG effector site for the IgG opsonic activity has been localized to residues 407-416 of the H chain of IgG1 and IgG3. This peptide sequence is now being

synthesized to verify its biologic activity.

The opsonically active moiety of C3 has been eluted from opsonized endotoxin emulsions. It is being characterized. The molecular interaction of C3b and the C3 inactivator is under investigation.

IV. Immunologic Tolerance and Autoimmune Disease

Autologous carrier-hapten induced tolerance is under study with regard to the cellular basis of its mechanism and its application to alteration of disease models, such as lupus-like disease in NZB mice.

V. Bone Marrow Transplantation and

Graft-Versus-Host Disease

A program of bone marrow transplantation in immunodeficiency disease, acute myelogenous leukemia, and aplastic

anemia is under way. It is helping to define minor histocompatibility loci as well as the cellular basis for graft-versus-host disease. Subpopulations of autocy-

totoxic lymphoid cells are being identified for clarification of the cellular kinetics of this subpopulation.

Division of Infectious Diseases

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Evaluation of New Antibiotic, Schering Corporation, \$10,000, Dr. Arnold Smith.

Sisomicin Treatment of Monkey Meningitis, Schering Corporation, \$5,000, Dr. Arnold Smith.

Cerebral Metabolism During Meningitis, Charles H. Hood Foundation, \$25,160, Dr. Arnold Smith.

Studies on Hemophilus Influenzae Type b, NIAID, \$147,412, Dr. David Smith.

Major Research Projects

I. Studies on a Vaccine for Hemophilus Influenzae Type b

This study is directed to the development of an effective immunogen for *H. influenzae* b (H.i.b). The reasons for considering active immunization vs. this bacterium are: it is the most frequent cause of bacteremia and bacterial meningitis among children, being responsible for 35% and 60% of such cases respectively, the incidence of these diseases has been increasing over the past 3-4 decades, so that at present there is a 1/500 risk of H.i.b meningitis per year among children under five years; the unacceptably high rates of mortality (5-10%) and neurological sequelae (30%) among survivors of H.i.b.

Earlier studies focused on polyribosephosphate (PRP) as the candidate immunogen. Work performed on earlier contracts and with funds from other sources defined techniques for purifying

PRP from (culture supernates of) H.i.b, methods, including RIA, of quantitating antibody (Ab), and the non-toxicity and immunogenicity of PRP for adults. Preliminary studies demonstrated a marked inverse relation between age and Ab response to PRP: children under 18 months produced Ab infrequently and at low titers.

Work performed during the past year further defined the anti-PRP Ab activity of healthy children, and the poor Ab response of infants to PRP. Vaccine was prepared for trial in Finland during an epidemic of meningococcal A disease. The results of a one year follow-up of that double-blind study demonstrate the efficacy of the vaccine for children over 18 months, and correlation of efficacy with immunogenicity.

Whole H.i.b, but not PRP, are immunogenic for rabbits. Furthermore, disease in infants promotes an Ab response. We have therefore sought the immunogenic principle of whole H.i.b. Culture

natants and 100Kxg pellets of outer-membrane-PRP complexes are immunogenic in rabbits. These materials contain high concentrations of endotoxin (LPS). Detergent precipitation and differential extraction of culture supernatant yields an immunogenic heterogeneous complex of PRP 5 polypeptides and trace quantities of LPS. Sub-fractions of this material indicate that the protein-PRP linkage is critical for immunogenicity. This complex raises Ab to PRP and to the proteins of the complex in adult humans.

We continue studies of techniques to produce this complex with better yields and of its immunogenicity in human infants.

II. Studies of an Animal Model for Bacterial Meningitis

Earlier studies demonstrated that Sprague Dawley rats have an age-related susceptibility to *H. influenzae* b meningitis that is independent of antibody status. Thus, pre-weanling rats develop bacteremia and meningitis histologically identi-

cal to human disease following intranasal inoculation of bacteria; weanling animals and adults are resistant to infection. This system has permitted study of the route of infection of the central nervous system, histologic effects of the disease and certain biochemical parameters. However, the size of the animals and the inability to obtain cerebro-spinal fluid does not permit certain types of studies, e.g. effects of the infection on cerebral blood flow and secondary consequences of impaired circulation on cerebral metabolism; relation of antibiotic kinetics in CSF to route of administration and dose.

The pathophysiology of bacterial meningitis has been incompletely described. The basis for the mental retardation commonly associated with this disease and improved or novel modes of therapy could result from a better definition of this pathophysiology. Such studies would be measurably aided by a suitable animal model of the disease.

Accordingly a primate model has been sought—and developed. Again, infant animals, but not adults, have been found to develop bacteremia and meningitis following experimental intranasal inoculation of *H. influenzae* b. This model has been systematically explored with the following salient findings. Intranasal inoculation of 10^7 bacteria is followed by colonization and evidence of nasopharyngitis. Bacteremia becomes evident at about 20 hours after intranasal inoculation with concentrations of 10^2 – 10^5 organisms/ml. Meningitis, evidenced by a CFS cellular response and positive cultures (generally 10^4 – 10^7 /ml), develops by about 48 hours after inoculation (range 18–144 hours). Detection of capsular antigen concentrations in serum lags behind positive bacterial cultures and exceeds those found in CFS by 10–100 fold. Affected animals show evidence of

systemic illness and generally (4/6 animals) develop pyoarthrosis. Having developed the model, the effects of the meningitis on cerebral blood flow and cerebral carbohydrate metabolism and antibiotic kinetics following intravenous and intraventricular inoculation are being evaluated.

III. Ampicillin Resistance of *H. Influenzae* Type b

All *H. influenzae* were sensitive to ampicillin until 1973 when resistant strains were associated with fatal cases of meningitis, and were found to constitute up to 10% of all *H. influenzae* isolated in Washington, D.C. Resistance to the antibiotic of choice has therefore prompted consideration of methods for assaying resistance, the basis for the resistance and alternative antibiotics for therapy of *H. influenzae* diseases. (The most effective, available antibiotic at this time is the potentially toxic chloramphenicol).

The ampicillin resistance of *H. influenzae* has been found to be produced by a degradative β -lactamase mediated by an extrachromosomal R factor. We have initiated studies to develop the technology to accurately and rapidly identify ampicillin resistant strains, survey their prevalence, define the molecular nature of the responsible plasmids, describe the properties of the β -lactamases, and evaluate alternative antibiotics for therapy of resistant strains.

An acidometric method of assaying β -lactamase has been modified and found to be uniformly accurate in identifying sensitive and resistant isolates within 30 minutes. Evaluation of all local isolates has revealed that approximately 5 and 10% respectively of type b and non-encapsulated strains are ampicillin resistant. Routine use of the enzymatic assay makes possible an early decision regarding therapy with ampicillin or

chloramphenicol.

Preliminary studies of the R factor of *H. influenzae* in other laboratories has suggested that they possess conjugal fertility among *H. influenzae*. We have confirmed this finding and have demonstrated that capsule inhibits conjugal transfer of the plasmid (which, in turn, probably explains why non-encapsulated strains recovered from Nature are more frequently ampicillin-resistant than encapsulated strains). Current studies are directed at defining the molecular nature of the conjugal apparatus and the relation of the *H. influenzae* R factors to those of enteric bacilli. Other studies are evaluating the *in vitro* activity of a number of antibiotics against panels of ampicillin sensitive and resistant strains. Potential candidates are to be tested in an animal model before use in the clinic.

IV. Etiology of Infantile Diarrhea

The recent documentation of a new class of viruses capable of inducing diarrhea in the young of man and many other species and of the production by certain *E. coli* of enterotoxins, some of which resemble cholera toxin physiologically and immunologically, has considerably advanced understanding of the etiology of infantile diarrhea.

We have examined the etiology of the diarrhea of infants admitted to the Children's Hospital Medical Center. No evidence of a role for enterotoxigenic *E. coli* was found unlike reports in certain epidemic settings; but approximately 40% of the infants had evidence of infection by the new (rheo-like) enteritis virus. We have also examined a panel of sera from several hundred children for antibodies to the rheo-like agent in an attempt to define the epidemiology of this agent and the role of systemic antibody as a resistance factor. Other studies have explored the relation of

enterotoxigenicity and the classic, serologic enteropathogenicity, and have found no correlation, raising questions regarding the value of the serologic test performed world-wide in diagnostic laboratories. Continuing studies are evaluating rapid methods of determining infection by these agents, their epidemiology in the community and in hospital, as a cause of nosocomial disease.

V. Rapid Diagnosis of Bacterial Disease by Immunologic Detection of Microbial Antigens

Evaluation of our clinical records has revealed that patients who have no primary disease and who develop a lethal bacterial disease generally die within 24 hours, and always within 48 hours after hospitalization or the onset of symptoms of a hospital-acquired infection. This time course often precludes etiologic diagnosis by conventional microbiologic laboratory methods; thus, therapy must be initiated empirically.

In order to diagnose life-threatening bacterial diseases rapidly and accurately, we have explored the immunologic detection of bacterial antigens in body fluids. We therefore initiated studies with polyribophosphate (PRP), the capsular antigen of *H. influenzae* b (H.i.b). This system was explored initially because of the importance of H.i.b diseases for our patients, our experience with this antigen and antibody to it, the active release of the antigen by bacteria *in vitro* and *in vivo* and the resistance of PRP to degradation by body fluids.

Initially, we studied the detection of PRP in spinal fluid and serum by counter-current immunoelectrophoresis and by inhibition of a radioactive antigen binding assay, and the utility of these techniques for rapidly providing an etiologic diagnosis. During these studies, we also found that quantitation of PRP in

serum correlated with clinical course: high initial concentrations and delayed clearance correlates with a poorer clinical prognosis.

Counter-current immunoelectrophoresis (CIE) is moderately cumbersome and frequently difficult to interpret when low levels of antigen are present. Agglutination of latex particles coated with antibody provides a sensitive and simple technique for the detection of (bacterial) antigens. The only major problem has been non-specific agglutination.

We have systematically investigated the detection of PRP with agglutination of antibody coated latex particles. By modifying existing procedures for coating latex particles with antibody, careful selection of antisera, and prolongation of incubation time, we have been able to increase sensitivity to detect as little as 0.1 ng/ml of PRP: this is 10 times more sensitive than CIE and equally or more sensitive than radio-immunoassay. We have been able to eliminate more than 95% of false (positive) reactions by adding non-immune animal serum to the latex particles (to absorb antibodies directed against the antiserum coating the latex particles) and by the use of a reducing agent in the reaction mixture (to dissociate the subunits of IgM antibodies directed against the animal serum). Any residual false (positive) reactions are identified by the use of control latex particles coated with non-immune animal serum.

We are currently evaluating this 30 minute latex agglutination test prospectively for the early diagnosis of meningitis and other infections caused by *H. influenzae* type b. In the laboratory we are comparing the results of latex agglutination with CIE.

Our modified latex agglutination assay appears to provide an extremely sensi-

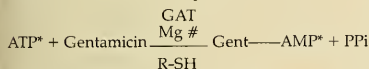
tive, reliable, and simple technique for the detection of *Hemophilus influenzae* type b capsular antigen. We plan to continue this work by applying this technique to other microbial antigens such as the capsular polysaccharides of *Pneumococcus*, Group B *Streptococcus*, *E. coli* K-1, *S. typhosa* and the enterotoxins of *V. cholera* and *E. coli*.

VI. Quantitation of Antibiotics by Enzymatic Assay and Application of These Assays to Evaluate Antibiotic Pharmacokinetics

A. Studies of the *in vitro* susceptibility of a panel of bacteria to a given antibiotic and limited evaluations in healthy human (usually adult) volunteers are the basis for statements regarding concentrations of antibiotics that are efficacious or toxic, and hence for dosage regimes. Few studies have been performed on the pharmacology of antibiotics in sick infants and children. Most of those have employed microbiologic methods that are time-consuming, relatively inaccurate and unwieldy — often requiring large volumes of serum. To overcome these practical problems and address the larger issues of the pharmacology of certain antibiotics in sick children, we sought improved assay technology.

An enzymatic method was developed for the assay of certain aminoglycoside antibiotics. The source of the enzyme was the bacterium *E. coli* infected with a R factor containing the genetic apparatus for the synthesis of an enzyme that inactivates the antibiotic(s). The basis for the assay is that the positively charged aminoglycoside and enzymatically altered antibiotic bind quantitatively to negatively charged cellulose. We have partially purified and described the properties of an adenylating enzyme specific for gentamicin and tobramycin. Using radiolabelled ATP, which does not bind to phospho-

cellulose, one can quantitate gentamicin in body fluids by determining radiolabel bound to phosphocellulose following incubation with the enzyme.



This assay which requires as little as 5 μl of specimen has proven to be rapid, sensitive, and more accurate than microbiologic assays; it has facilitated detailed pharmacologic investigations aimed at determining correlations of dose with serum concentrations, and serum concentrations with efficacy and toxicity.

1. Children require a larger dose of gentamicin than adults in order to obtain similar peak serum concentrations. Dosage may be improved either by giving children larger doses calculated on the basis of body weight or by calculating the dosage on the basis of body surface area.

2. Although marked individual variation on peak levels occurs, a given patient tends to maintain similar peak levels. The amount of individual variation is less when dosage is based on surface area.

3. The half-life of gentamicin is short (mean: 75 min; range 26-230 min.). Individuals who have fever and anemia and who receive gentamicin IV have particularly short half-lives.

The last observation suggests that a patient receiving gentamicin with an eight hour dosage interval may have subtherapeutic gentamicin levels for up to 6 hours during each 8 hour period. The occurrences of breakthrough bacteremia, usually just before a gentamicin dose, in 5 patients suggests that this may be clinically significant.

We are therefore currently comparing the efficacy and toxicity of conventional doses of gentamicin (60 mg/M² q 8 hours) and high doses of gentamicin

(60 mg/M² q 4 h) in a controlled study of febrile, granulocytopenic cancer patients who are initiated on empiric antibiotic therapy.

All patients will have: 1) complete pharmacologic evaluation, 2) careful monitoring for toxicity (serial audiograms, creatinine clearances, electrolytes), 3) careful assessment of therapeutic response (rate of clearance of bacteria from blood or focus of infection, duration of fever, fever index).

All bacteriologic isolates will be examined in the laboratory to determine their sensitivity *in vitro* to the antibiotics used.

It is our aim to: 1) compare the relative efficacy and toxicity of these three antibiotic regimens, 2) correlate efficacy and toxicity with serum antibiotic levels and *in vitro* sensitivity of the patient's pathogen.

B. New enzymatic assays have now been developed for chloramphenicol and amikacin, a new aminoglycoside antibiotic active against many bacteria resistant to gentamicin and tobramycin.

The chloramphenicol assay employs a novel chloramphenicol acetyl transferase produced by an R factor recovered during earlier studies. This enzyme has been purified 120 fold, characterized, and developed as a clinical reagent. The assay depends on the unique property of Millipore membranes to quantitatively bind chloramphenicol and its derivatives, and employs radioactive acetyl coA as acetyl donor. The enzyme attacks only chloramphenicol and not natural derivatives, and is not inhibited by natural products or other antibiotics. Initial pharmacokinetic studies in infected infants treated with chloramphenicol have revealed that peak serum concentrations vary markedly: bloodstream clearance of the drug appears to be an extremely sensitive index of liver function; and may be prolonged in certain children with no underlying

disease.

An acetylase mediated by another R factor and which attacks gentamicin, kanamycin, amikacin, and tobramycin has also been partially purified. It can be assayed as above, and is being adapted for clinical studies.

VII. Bilirubin Binding by Pediatric Formulations of Gentamicin

Sulfonamides have been found to reduce serum bilirubin and increase brain bilirubin concentrations, and, in some instances, cause kernicterus in human infants. This adverse activity results from interference of bilirubin binding to serum albumin and the cerebral toxicity of bilirubin. It was therefore very concerning when pediatric formulations of gentamicin, an antibiotic used commonly to treat bacterial infections in newborn infants, was also reported to interfere with *in vitro* bilirubin-albumin interactions. No evidence of clinical kernicterus was correlated with gentamicin usage.

Studies in this laboratory have resolved an interesting riddle. Pure gentamicin does not affect *in vitro* bilirubin binding to albumin in pools of newborn serum when assayed in each of 3 systems. However, the clinical formulation does affect binding. Testing each of the components of the formulation incriminated one of the preservatives, methylparaben, as the component active in interfering with bilirubin-albumin binding. These studies are being pursued further in Gunn rats. These results have led to a reformulation of the manufacturer's preservatives; they may have broader application since parabens are used commonly as preservatives in many pediatric drug formulations.

Division of Nephrology

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Financial Support:

Immunologic Mechanisms in Renal Diseases in Childhood, NIAMDD, \$150,488, Dr. Rosen.

Trace Element Abnormalities in Children with Chronic Renal Failure, NIAMDD, \$50,627, Dr. Grupe.

Major Research Projects

I. Mechanism of Glomerular Injury by Immune Complexes

The major laboratory model used is Hemann's nephrosis in rats (Autologous Immune Complex Nephritis), where immunization with autologous, homologous, or heterologous tubular antigen leads to the glomerular deposition of circulating immune complexes and the development of proteinuria. Previous work has established that an antibody directed towards antigens in the apical portion of the proximal tubule is responsible for the formation of disease producing complexes. Several avenues are currently under investigation.

The anatomic location of the tubular antigen is being determined by immunohistologic and ultrastructural techniques, to define whether the antigen resides in the brush border or in cytoplasmic reabsorption droplets. This is of importance, since disease producing antigenicity could be a function of reabsorption and alteration of a glomerular filterable protein, produced in some other organ. We have produced evidence by immunofluorescent techniques that antigenic determinants in the proximal tubule are

shared by lung and small intestine, although their shared antigens, unaltered, are incapable of participating in the formation of glomerular deposits. This is being re-examined.

An inbred strain of rat, Foreman A-2, has been developed that is hyporeactive to native autologous antigen but reacts normally to antigen altered by sulfonation. This strain is being used to determine T and B cell reactivity, and the genetics of the reactivity to tubular antigen.

The influence of immune complex deposition on proteinuria is being investigated ultrastructurally in collaboration with Dr. E. Schneeberger in reactive and hyporeactive rat strains, and functionally by ultrastructural immunohistology and by alterations in the clearances of PVP and dextrans of varying molecular weight.

The role of complement production and turnover is being investigated in an *in vitro* liver cell system jointly with Dr. Harvey Colten and the renal regulation of hyperlipidemia in the nephrotic state is being explored, each using the Hemann nephrosis model.

II. Therapy of Glomerular Disease

The use of chlorambucil in the therapy of

glomerular diseases has been under investigation for 10 years. Results clearly show chlorambucil to be of benefit in minimal lesion nephrotic syndrome that is steroid resistant, steroid dependent or frequently relapsing on steroids. Current trials are designed to evaluate the dose response in this group of patients as well as more accurately determine both short and long term side effects, particularly gonadal function. The effect of chlorambucil on other glomerular diseases is also under investigation; with some evidence of effect in focal segmental glomerulosclerosis; focal global sclerosis and anaphylactoid purpura nephritis with diffuse proliferation, epithelial crescents and the nephrotic syndrome. There is insufficient data yet to determine effectiveness in membrano proliferative and mesangial proliferative glomerulonephritides.

The effectiveness of dose and duration of therapy with steroids in minimal lesion, steroid responsive nephrosis is being explored in a controlled protocol. Also the effectiveness of high dose methylprednisolone "pulse" therapy is being evaluated in rapidly progressive glomerulonephritis, membrano proliferative glomerulonephritis and lupus nephritis.

III. Studies on Hypertension

The etiology and epidemiology of hypertension in children following renal transplantation is being investigated with regard to relative renal ischemia, renal plasma flow, the renin angiotensin system, the influence of the primary renal disease and the effect of pre-transplant native kidney nephrectomy.

The effectiveness of clonidine, minoxidil and propranolol in the management

of hypertensive children, with and without parenchymal renal disease, is being evaluated.

IV. Nutrition and Trace Element Balance in Chronic Renal Insufficiency

The role of energy balance and protein turnover is being evaluated in patients with chronic renal insufficiency and growth failure with estimation of the kinetics of energy and protein utilization,

establishment of minimum requirements and efficiency of utilization. Studies of trace element balance indicate a depletion of both copper and zinc in chronic renal insufficiency that antedates hemodialysis and is neither connected nor aggravated by dialysis. The role of zinc balance in appetite (and, hence, total energy balance) is under investigation in this group of children, as well as in laboratory animals.

Division of Newborn Medicine

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Edward Lawson, MD, Ann Stark, MD, Ilene Sosenko, MD

Financial Support:

Pulmonary Surfactant Regulation in Fetus and Newborn, NICHD, \$75,410, Dr. Taeusch.

Bile Acid Metabolism in Infancy and Childhood, NICHD, \$139,305, Dr. Watkins.

Periodic Breathing and Apneic Spells in Premature Infants, Hood Foundation, \$19,000, Dr. Taeusch.

Glucocorticoid and Hyaline Membrane Disease, The Upjohn Co., \$12,500, Dr. Avery.

Comparative Study of Sweat Test Methodologies, Cystic Fibrosis Foundation, \$17,205, Dr. Avery.

Major Research Projects

I. Respiratory Distress Syndrome and Lung Surfactant

A. Clinical Studies. Women in premature labor, at risk of giving birth to infants with RDS, are being treated with I.M. corticosteroids in a controlled therapeutic trial. The thrust of this study is to assess the benefits of prenatally administered steroids in a carefully controlled large, double blind study. Long term follow up of enrolled infants will assess long term effects of corticosteroids, if any, on growth and development.

Two other clinical studies investigate chronic pulmonary disease in infants surviving the acute phase of RDS. Since Vitamin E is a known physiologic antioxidant, and since many infants with RDS appear to develop long term symptoms of oxygen toxicity, a controlled trial of I.M. Vitamin E therapy in patients with RDS is being conducted.

In collaboration with Dr. Mary Ellen Wohl, a technique to evaluate pulmonary airway flow limitation in infants is being developed. It is hoped that this will assist in evaluating the long term changes in small airways that a number of RDS sur-

vivors appear to suffer from.

B. Animal Studies. Evidence from previous studies suggests that adrenergic agents and also changes in lung volume may stimulate the type II cells of the lung to release surfactant into the alveoli. Therefore, several projects are designed to investigate these non-synthetic factors that may be regulating the maturational appearance of surfactant in the lung. Sheep fetuses are infused with sympathetic agents and tracheal fluid is continuously collected for measurement of surfactant activity. In another project, fetal rabbits, with low pulmonary surfac-

tant activity, are delivered and treated with positive end expiratory pressure, to alter lung volume, in order to assess the effect on lung compliance and surface tension.

Recent evidence from clinical studies suggests that infants of diabetic mothers are at greater risk of developing RDS due to a factor associated with diabetes which appears to inhibit lung maturation. An animal model for diabetic pregnancy in alloxan treated rabbit does is being developed to study the effects of maternal hyperglycemia on the fetal surfactant system.

II. Developmental Gastroenterology

The overall objective of these investigations is to define the development of bile acid synthesis, secretion and the regulation of bile acid kinetics in childhood. This information will be used to characterize the developmental aspects of fat absorption and to explore the mechanisms responsible for the abnormalities of the intraluminal phase of fat absorption in premature infants. The investigations to date have examined the regulation of bile salt pool size and synthesis rate in fullterm infants and more recently in premature infants utilizing a stable isotope dilution technique and deuterium labeled bile acids.

Information recently reported extends the initial observations reporting a reduced bile salt pool size in fullterm infants, to premature infants. A marked heterogeneity was observed, so that the infants whose mothers had been treated with dexamethasone or phenobarbital, exhibited a marked increase in bile salt pool size, together with a corresponding increase in intraluminal bile acid concentrations and an improved efficiency of fat absorption. These preliminary data present the possibility that prenatal factors may significantly alter functional maturation of the liver and intestinal tract in the

young infants and thus must be considered to be of major significance to the nutritional status of the developing premature infant. Studies are now in progress to further characterize the influence of diet on bile acid kinetics and fat absorption in the small premature infant.

A. Metabolism of Lithocholic Acid in the Fetus. A second area of investigation concerns the excretion of maternally derived secondary bile acids in the human fetus. Having established that the human fetal liver principally excretes deoxycholic and lithocholic acid acquired by placental transport from the mother by a process of conjugation with glycine or taurine and this sulfation at the 3 hydroxyl position; an animal model system utilizing near term fetal sheep was devised and characterized. Investigations are now in progress to examine the excretory pathways and metabolism of these bile acids in the fetal sheep. These studies should provide a means for investigating in the fetus, hepatic excretory mechanisms and provide a means to evaluate the possible hepatic toxicity of maternally derived lithocholic acid.

B. Metabolism of Intralipid by the Premature Infant Investigations have been initiated in collaboration with Dr. Frank Franklin and Dr. Jan Breslow, characterizing the metabolism of an intravenously administered triglyceride emulsion (Intralipid) in 14 premature infants. It was found that the premature infant metabolizes and utilizes the triglyceride emulsion efficiently, however, all infants became hypercholesteremic. The hypercholesteremia was principally as free unesterified cholesterol which was present in the serum bound to a lipoprotein of pre beta migration. An animal model was then designed to evaluate this phenomenon further, and studies are in progress to evaluate the mechanisms of chole-

sterol metabolism during Intralipid infusion. Accordingly, it will then be possible to examine in detail the sequences responsible for cholesterol accumulation and the apparent interruption in cholesterol ester formation observed in the premature infant.

II. Respiratory Control and Neonatal Apneic Spells

The thrust of this research is to determine what causes neonatal apneic spells. The underlying hypothesis is that some aspect of neural control is immature in these infants making them at risk for episodic apneic spells.

Two new therapeutic approaches to treating apneic spells are currently in use in many nurseries—positive and expiratory pressure and oral aminophylline. One clinical study in progress is aimed at actually assessing the effectiveness of aminophylline in preventing spells in a controlled double blind fashion. CO₂ response and other aspects of respiratory control are evaluated in these infants, on and off aminophylline, in order to seek its therapeutic mechanism.

In a second study, the central influence of vagal pulmonary volume receptors on rate and depth of breathing is being investigated in infants with and without positive end expiratory pressure.

A third study assesses the neural and mechanical factors contributing to maintaining upper airway patency during sleep. The interaction of these factors in producing the airway obstruction which occurs with changes in neck position in sleeping infants is being examined.

Division of Pulmonary Diseases

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Financial Support:

Respiratory Mechanics in Children, NHLI, \$42,000, Dr. Wohl.

Measurement of Pulmonary Function In Infants and Children By Transient Forced Oscillations,
\$84,499, NHLI, Cambridge Collaborative, Inc., Drs. Strieder/Wohl.

Major Research Projects

I. Exercise Physiology

Our previous studies of children with congenital or acquired heart disease were focused on analyzing the abnormal modality of pulmonary gas exchange. Another aspect of that work was to demonstrate the adaptability of respiratory control mechanisms to widely varying ventilatory requirements, not only as a function of work load but also as a function of lung performance. Whenever the efficiency of gas exchange is impaired, as by a large right-to-left shunt or by pulmonary vascular disease, exercise ventilation rises to much higher values than predicted for a given work load. Current knowledge of control of breathing during exercise cannot explain this "fine tuning" of the steady state ventilatory response. Faced with similar difficulty in accounting for the hyperventilation induced by experimental metabolic acidosis, Lambertsen advanced the hypothesis that a "second central chemoreceptor", unprotected by the blood brain barrier, increases respiratory drive in response to systemic metabolic acidosis. Such a receptor could provide the booster stimula-

tion required to drive the respiratory system at very high ventilations, with lactic acid contributing the added stimulus. Another possible pathway for a lactate stimulus is in the carotid body which is responsive to arterial blood acidosis. This "peripheral" chemoreceptor, however, may be insufficiently sensitive, as pointed out by Lambertsen in relation to experimental acidosis and Lugliani in human exercise physiology.

The Lambertsen hypothesis provides a satisfactory mechanism to account for the abnormally high respiratory drive observed during exercise in children with congenital heart disease. The hypothesis that CNS structures, other than the known medullary chemoreceptor and ponto-medullary respiratory centers may exist is supported by the observation that a boy with hypothalamic dysfunction had exercise hypoventilation, whereby his ventilatory response to exercise followed his ventilatory-PCO₂ response curve.

We propose to test the applicability of the Lambertsen hypothesis to exercise physiology. We look at blood lactate level as a messenger from muscle to brain, which converts muscle need for oxygen

into a H⁺ stimulus. Blood lactate level seems to be adjusted to muscle needs by intervention of the Bohr effect in muscle capillary. We surmise that inhibition of carbonic anhydrase, by interfering with CO₂ transfer from muscle cell to muscle capillary, may induce abnormally high muscle PCO₂ and substitute respiratory for metabolic acidosis to drive the Bohr effect. Lower blood lactate levels would then be reached allowing us to observe the effect of altering exercise metabolic acidosis on ventilatory control mechanisms. There is an additional advantage to selecting a carbonic anhydrase inhibitor (acetazolamide) for study. Acetazolamide is used in healthy young people to prevent acute altitude sickness, whereas the effect of the drug on exercise performance is poorly known.

The method for assaying carbonic anhydrase in blood has been developed. Results show that 500 mg of acetazolamide in a single intravenous dose, induces 50% inhibition of blood carbonic anhydrase. The effect on exercise ventilation is small and demonstrable only for high work loads. With untrained volunteers, whom we have studied so far, care must be taken to rule out the influence of

repeated tests on the same day (fatigue), on successive days (muscle pain) or after several weeks (training). Once the optimal dose of acetazolamide and optimal exercise protocol have been determined, we will call in trained volunteers whose performance should be more easily reproducible.

Traditionally ventilation is measured as the product of tidal volume by frequency and it is well known that both increase during exercise. Another way of measuring ventilation, proposed by Milic-Emili, considers the product of mean inspiratory flow rate (V_i) by the fraction of time spent breathing in; the latter is calculated as the ratio of duration of inspiration to duration of total breathing cycle. This type of analysis when applied to exercise shows that most of the increase in ventilation results from increased V_i whereas the timing of inspiration and expiration is unchanged in normal individuals. We surmise that the ratio of inspiratory time to total time may provide information on mechanics of breathing during exercise, whereas V_i is a function of respiratory drive.

II. Respiratory Mechanics in Children
Mechanical properties of the lung in normal children 7 to 16 years of age are being investigated; a technique to determine maximum expiratory flow in young infants is being developed; and studies to attempt to define the sequelae of congenital lobar emphysema have been initiated.

The elastic properties of the lung change with age and from the age of 7 to 16 years, the lung appears to become stiffer. Maximal expiratory flow is determined by the elastic properties of the lung and by the resistance of the airways. Great variability in maximal expiratory flow volume relationships have been observed in adults and in children. Longi-

tudinal studies in approximately 20 children are underway to elucidate the relative role of the changing elastic properties of the lung (obtained from static pressure-volume curves of the lung) and the size of the airways (estimated from measurements of anatomic dead space) upon maximum expiratory flow volume relationships.

In contrast to the older child, infants will not make a forced expiration on demand. We have developed a technique of obtaining "forced" expiration or maximal expiratory flow by applying a rapid transient of positive pressure around the infant's chest to stimulate the pressures he can make with his expiratory muscles. We have shown that at a given lung volume below resting and expiratory lung volume, flow is maximal in that further increases in pressure applied to the chamber and hence around the lung, produce no further increases in flow. We have shown that the ventilation of normal newborns is not limited by the mechanical properties of the lung and that they can increase expiratory flows to more than 3 times that observed during quiet breathing. We are currently applying this technique to infants recovered from mild respiratory distress and recovering from bronchopulmonary dysplasia, to determine the extent to which abnormalities in mechanical properties of the lung may limit ventilation.

Studies are currently underway to attempt to define the residual abnormality in patients who have undergone excision of a lobe in early childhood for congenital lobar emphysema. Preliminary studies in 6 children who are now over 8 years have shown evidence of airway obstruction. However, studies of the distribution of ventilation and preliminary blood flow using Xenon 133 radioscintigraphy have shown no abnormalities

and the expected gravity dependent gradients of perfusion and ventilation have been maintained. The severity of the airway obstruction contrasts with the normal gas exchange and our current studies are designed to better locate the site of obstruction and the physiologic consequence of this obstruction in terms of gas exchange.

III. Measurement of Pulmonary Function in Infants and Children by Constant Forced Oscillations

The properties of infants' and young children's airways will be analyzed by the transient forced oscillation technique. An acoustical transient (a noise similar in intensity to a clap) is caused to propagate into the airways. By analysis of the transient pressure wave forms incident upon and reflected from the airways, quantitative information regarding the respiratory system can be obtained. During the current year, the theoretical background for this measurement has been expanded; the source of the acoustical transient has been developed (it must have a frequency content from 1 to 10,000 Hertz); the wave tube in which the acoustic impulse is propagated has been designed and built; methods of coupling to the airways have been developed; and the computer facilities to perform the Fourier transform algorithms have been established. This technique should be a safe, non-invasive method of measuring airway resistance and reactance over a wide range of frequency. In addition it may be possible to estimate the geometry of at least the upper airways and the first few generations of bronchi from these measurements.

Division of Services to Handicapped Children

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Financial Support:

Harvard-MIT Rehabilitation Engineering Center, SRS, \$62,000, Dr. Berenberg.

Charles A. Dana Foundation, Inc., \$100,000, Dr. Berenberg.

The Hearst Foundation, Inc., \$50,000, Dr. Berenberg.

The United Cerebral Palsy Research and Education Foundation, Inc., \$200,000, Dr. Berenberg.

Hoffmann-LaRoche Company, \$8,940, Dr. DeLuca.

Liberty Mutual Insurance Company, \$5,000, Dr. DeLuca.

Major Research Projects

I. Gait Analysis

Current research endeavors are concerned with the study of normal and pathological gait. The primary purpose of this work is to develop methods and techniques that will be useful for determining the etiology and evaluating current treatment modalities of locomotor disorders incurred by various neuromuscular-skeletal diseases, i.e., cerebral palsy, muscular dystrophy, hemiplegia, degenerative arthritis, etc. Such evaluation is being considered from a basic bioengineering standpoint and includes the development of data acquisition and data analyses systems, for measurements of body movements, muscle activity, and forces developed during gait. These data are being studied by considering basic measured and calculated biomechanical parameters of gait, for the whole body as

well as each limb segment, and by more complex bioengineering considerations of modeling both normal and pathological gait and the effects of abnormal muscle behavior.

To accomplish the laboratory's long term objective, the goal for the present year has been pilot studies of: (a) 20 normal children and (b) 15 children with cerebral palsy to serve as empirical models for the development of the laboratory system. From these studies, progress in the establishment of the appropriate laboratory system has consisted of:

1. Data Acquisition. This is accomplished using the gait lab's 40' walkway with simultaneous synchronized 8 channel EMG, force plate and film recording. Electronic and computer programming systems are in the final stage of development and require further effort to refine an efficient real-time system, which can handle the young and handicapped child

easily, and accurately and which can provide a readily accessible data storage bank.

2. Data Analysis. Systems have been developed for providing three dimensional motion analyses of the lower extremities from movie film and storage and display of this from the computer system. The computer system can also display the EMG and force plate data as well. Current efforts are concentrated on: (a) three dimensional analysis of the trunk and pelvis, (b) error analysis of the system, and (c) integration functions of velocity and displacement of the dynamic center of gravity from the force plate data.

Further work is planned for obtaining body segment velocities and accelerations by differentiation of the motion analysis. In addition, integration of all the programs for both data acquisition and data analysis into a comprehensive computer system for the use of non-engineering medical personnel is planned.

II. Monitoring, Modifying and Testing Anterior Spinal Instrumentation

The use of the Dwyer technique and instrumentation for anterior spinal fusion in handicapped children with scoliosis is an accepted procedure and offers great promise of managing this most difficult problem. Complications associated with its use may be avoidable provided adequate quantitative knowledge about certain biomechanical characteristics of it is gained. Sufficient engineering technology is available to develop a sufficiently small biotelemetric device which will provide the basic quantitative information on the forces and stresses on the apparatus which will enable solutions to these problems to be found.

The specific aims of this study are:

1. To develop a small, simple biotelemetry device which is biologically compatible and which will provide the information concerning the dynamic tension forces in the Dwyer Cable.

2. To use this device to determine the relative tension forces produced in the cable during its intraoperative insertion.

3. To use this device to determine the relative change in tension forces on the cable during the postoperative period.

4. To ascertain the change in relative tension forces in the Dwyer Cable that occur with time in an attempt to predict the length of time to interbody fusion.

Since the initiation of this study, the protocol has proceeded along in the following manner:

1. Development of biotelemetric apparatus

2. *In vitro* testing of apparatus

3. *In vivo* testing of apparatus

4. *In vivo* human implantation of the apparatus.

Development of a biotelemetric device to measure the tension forces in the Dwyer Cable has been completed. An

inductively-powered biotelemetry unit contained within a load cell mounted on the cable is utilized. Changes in cable tension cause deformations of the load cell sensed by a semiconductor strain gauge. Changes in the resistance of the strain gauge, in turn, cause alterations in the modulating frequency of the telemetry unit. Shifts of approximately ten Hertz per pound of tension are obtained in practice. The telemetry unit amplitude modulates the incident electromagnetic field and is detected across the primary of the power coil. The implant is driven externally by a tapped coil — designed to fit the animal or patient as a belt. The loaded gauge is calibrated by comparison with an unstrained reference strain gauge. The calibration, which can be performed at any time, is obtained in the presence of a DC magnetic field operating an internal reed switch. The electronics, with the exception of the ferrite core antenna, is hermetically sealed inside the titanium load cell. The antenna coil is designed to imbed within a high-density ceramic cup whose open end is epoxy-sealed over the titanium capsule.

Seven tension monitoring telemetric devices have been produced. Successful testing and calibration of all seven units over a range of temperatures from 25-40°C have been performed. The results have indicated that the monitoring system produces accurate and repeatedly reliable results independent of temperature over the physiological range tested.

Before human implantation is to be performed, a study has been planned and is currently in progress for animal testing of the monitoring system.

Seven mongrel dogs have been instrumented over three vertebrae after excising two intervertebral discs and packing bone graft. One dog died in the early postoperative period from infection. The

remaining six dogs are alive and well and the intraoperative, early and late postoperative tension monitoring have been and are currently being performed.

This *in vivo* testing with dogs indicates a rapid accommodation of tissues and vertebral structures to initial tensions applied in the Dwyer technique. Within hours, a small percentage of the initial 80 lbs. or more tension remains. Furthermore, manual bending of the animals about several axes produces high tensions. These approach initial tensions with simple spinal extension and especially with extension in the left sagittal oblique plane. Flexion in this plane of maximum mechanical advantage logically results in a decrease in measured tensions, although small.

As the project continues, the monitoring device will remain implanted for a minimum of six months. During this time, monitoring will be performed periodically, and at the end of the prescribed period, the animals will be sacrificed and relevant data taken. Mass and volume of each implant will be compared with starting values, descriptions of the tissue area and capsule made, complete tissue histology done, and status of the transducer electronics investigated.

III. An Objective Clinical Measure of Spasticity

This project has as its goal the development and evaluation of a clinical device for objective measurement and classification of spasticity based on patterns of force, displacement, and EMG.

The project entails:

1. The construction of a device for the controlled rotation of the forearm (supination-pronation) with instrumentation for measuring muscular activity and reaction forces.

2. The evaluation of the design on a pool of normal subjects and hemiplegic

cerebral palsied patients so that necessary refinements can be made to the device.

3. The design and implementation of automatic data analysis schemes to derive the most meaningful, clinically useful parameters from the families of force-displacement-EMG curves.

4. Actual evaluation of treatment of patients correlating the new objective measures with conventional clinical evaluation of progress.

All experiments involve controlled pronation of the the forearm. They are currently being extended to include supination. Seated upright in a dental chair, the subject grasps a spade type instrumented handle. An elbow cup supports the subject's forearm and serves to align his ulna with the axis of rotation of the handle shaft.

Movement waveforms include displacement steps and ramps of different velocities. Excursion is to 45° from the vertical. The subject is asked to maintain a pre-stretch isometric contraction in the supinators and the timing of the controlled motion is randomized to minimize prediction and adaption. The initial condition was varied by using different levels of pre-stretch bias force. Non-static conditions were also tested where the subject again maintains a pre-stretch bias force but with the handle now moving at a slow speed in the direction of supination.

Recorded measurements include arm angle torque at the handle and raw and integrated EMG of the biceps brachii. Force versus stretch diagrams are plotted on an X-Y plotter.

The current status of the project is at the point of turning from hardware and tests on normals to software development for analysis and preliminary tests on patients. We have selected as the initial group to be studied twelve to twenty-

four adolescent and young adult patients from the cerebral palsy clinic who have indicated a willingness to participate. These individuals appear to be excellent subjects for the research design since they are old enough to be cooperative study subjects. In addition, their involvement is unilateral and the contralateral uninvolved extremities provide opportunity for control data for each subject. The emphasis in the tests will be on determination of range of movement, stiffness, and the non-linear, speed dependent aspect of resistance to motion.

Experiments on these patients will employ trapezoidal displacement waveforms of various speeds and amplitudes. This will help establish for each subject a range of reasonable stimulus speed and amplitude that would be used for therapeutic assessment. The effect of therapy on the relative dominance of dynamic versus tonic sensitivity to stretch can also be studied. Sinusoidal rotation will also be applied to study frequency response and phase characteristics. Subjects will also be asked to perform voluntary pronation and supination and EGM records of antagonistic muscles will be studied for the extent of the loss of reciprocal innervation and changes in this aspect of spasticity will be correlated with variations on other parameters as a result of treatment.

IV. TIPSCHE: A Tactile Information Processing Substitution Channel for Hearing TIPSCHE is a study of the feasibility of assisting hearing-impaired and deaf children in processing auditory information through the use of the tactile sensory system. The channeling of some or all auditory information through the tactile system requires achieving several objectives:

1. Design and fabrication of systems to receive and process multiple signals of

varying importance, varying informational content, and varying acoustical characteristics.

2. Appropriate output transducers and their interface with the body surface.

3. Fabrication and evaluation of preliminary systems using both normal-hearing adults with simulated deafness and deaf adults, and involving both laboratory studies and field studies.

4. Clinical evaluations on children following necessary modifications.

The final objective is the clinical introduction of tactile-display devices. They must be routinely wearable for long periods by infants as well as older children and adults, and must present information interpretable by the user as representing his meaningful acoustic environment. Although devices that aid speech communication and the development of language competence are clearly the most important (and thus constitute our ultimate goal), we will also consider devices that are oriented toward the appreciation of nonlinguistic information.

Three lines of work have been pursued. The first is the design and development of a portable "simple aid", comprising a single tactile transducer delivering a modulated single frequency. The amplitude of the carrier frequency varies as the waveform of the acoustic input. We are concerned with variables of transducer characteristics, vibrator carrier frequency and modulation scheme, skin location and transducer mounting, and size and weight of the aid. A laboratory model has been designed and fabricated and is undergoing tests. Later, the design will be modified to achieve portability. The device has an audio bandwidth of 300-6000 Hz. The envelope of the acoustic signal is compressed and used to modulate a 250 Hz. frequency driving a hearing aid bone oscillator (Radioear

B70A). The current system has approximately a 20 dB dynamic range with a modulation bandwidth approximating 50 Hz. Transducer location will be systematically explored. Some modifications of the system will be made to reduce power requirements and increase portability.

The second area of work is analysis of a method of perceiving speech used by deaf-blind persons and called the "Tadoma Method". The recipient places a hand or hands on the speaker's face and monitors certain features of the speech-articulation process. The Tadoma Method demonstrates the possibility of perceiving speech through the tactile sense. A research plan has been developed for subsequent work comprising: (1) a survey to determine incidence of use, particulars of training procedures, and estimate of success; (2) interview and observation of Tadoma users to determine which articulatory parameters are monitored, procedures used in familiarization with a new speaker, and estimates of success; and (3) some laboratory studies on performance under systematic control of stimulus materials and speakers.

The third area of work is the preliminary development of a laboratory tactile-display system, comprising a third octave filter bank, computer, and an Optacon tactile-display transducer. The hardware interfacing the computer with the transducer has been developed and software has been developed for presenting specified vibratory patterns under computer control.

V. EMG Research

A. *Analysis of the Electromyographic Signal.* The electrical manifestation associated with innervation and contraction of skeletal muscles is under analytical study with the goal of establishing rational mathematical models useful in both diagnostic and control applications

of electromyography. Based on previous work performed in our laboratory, a mathematical model has been developed for the EMG signal recorded during constant force isometric contractions.

B. *Force-EMG Signal Relation for Isometric Contractions.* A study of the relationship between the EMG signal and the isometric force output of three human skeletal muscles is being performed on healthy adults. The possible dependence of the EMG-force relationship on functionally different muscles such as the first dorsal interosseous, biceps brachii, and deltoid, and on different forms of exercise is being investigated. The statistical and physiological properties of single motor units during varying force and force-rate contractions will be investigated and modelled mathematically according to random-processes analysis approaches that have proven successful for isometric constant-force contractions.

The purpose of this study is a) to provide a basis for relating the EMG signal to the force output of human skeletal muscles, and b) to describe some physiological properties of the motor units in normal muscles. This study will also provide a non-invasive technique and baseline data which can be used to quantitatively measure the difference between the performance of normal or abnormal muscles and thus should result in a useful clinical implement for evaluating the state of a muscle.

C. *Computer Simulation of the EMG Signal.* The current mathematical model of the EMG signal that has been developed in our laboratory is being implemented on a digital computer. This will allow us to generate EMG signals associated with any given force level and time duration of a muscle contraction. Such an approach has the advantage of providing test EMG signals for developing processing and

analysis techniques without performing long, involved experiments with human subjects.

D. *Classification of Local Severity of Spastic Muscles by Electromyographic Investigation.* This study attempts to describe and classify the degree of severity to which the muscles in the upper limbs are afflicted in cerebral palsy patients by analysing the EMG signals from these muscles while the patient attempts voluntary movements. Special attention is given to the amplitude and phasic relationships of the EMG signals.

E. *Myo-feedback Device.* A compact, lightweight EMG signal detector with audio output is currently being designed and constructed. The device consists of a velcro strap which holds the recording electrodes positioned over the muscle of interest, and a small box containing the battery and circuitry worn on a belt around the waist. The device emits a sound whenever the EMG signal from the desired muscle surpasses a pre-set level. In this fashion the patient is aware that the muscle of interest is active. It is anticipated that the myo-feedback device will be useful in exercising and developing atrophied muscles as well as improving the gait pattern of patients afflicted with neuromuscular diseases by providing a surrogate feedback for muscle awareness.

F. *The Effect of Valium on the Normal EMG Recorded from the Deltoid Muscle.* In this study we are investigating whether the medication, Valium, has any direct effect on skeletal muscles. It is a well known fact that Valium is a central nervous system depressant and hence indirectly acts as a muscle relaxant. We are performing experiments to record and analyze EMG signals from skeletal muscles during constant force isometric contractions. Preliminary results indicate a

possible direct action on the muscle.

G. Investigation of the Degenerating Process of Severed Nerves Surrounded by Cloth Tubes. Tubes constructed of Dacron or Teflon knitted cloths are fixed around the distal end of severed peripheral nerves. We are interested in investigating the degeneration of the enclosed nerve fibers and the growth of connective tissues and fatty tissue between the nerve and the tube as well as outside the tube. These developments are being studied as a function of implantation time, cloth material, cloth porosity, and the space between the tube and the enclosed nerve. The purpose of this study is to obtain the parameters of the tube that will afflict minimal damage to the severed nerve.

The tube will be used to house electrodes for recording nerve signals and form an electrical interface between the nerve and external prosthetic devices.

H. Chronically Implantable Electrode for Recording Nerve Signals. We are in process of optimizing the design of a chronically implantable electrode for recording voluntarily elicited nerve signals from truncated nerves for the purpose of interfacing nerve signals to apparatus for controlling multiple-degree-of-freedom prostheses. The electrode has been constructed so as to have a minimal detrimental effect on the nerve, a maximal signal-to-noise ratio, and to be implanted around the nerve with a relatively simple procedure. The current version has been

implanted around the sciatic nerve of rabbits. Nerve signals have been recorded for a period of 3 months. We are now preparing to implant a modified version of the electrode in primates.

I. Acquisition of Functionally Distinct Nerve Signals on the Surface of Nerve Trunks. This project is designed to investigate the feasibility of recording distinguishable signals from the surface of large nerve trunks and directly associating the signals with specific muscle functions. It is hoped that two or three functionally distinct signals can be obtained from one nerve trunk such as the median nerve. It would then be possible to obtain near-natural control of prosthetic devices.

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Major Research Projects

I. Clinical and Physiologic Assessment of the Outlook for Children with Congenital Heart Disease

One of the principal efforts, now being completed, is a *national cooperative study of the long range clinical course* of patients with congenital pulmonary stenosis, aortic stenosis, and ventricular septal defect. The results, based on data obtained from over 2,000 patients, followed up for 19,000 person years, describe the expected clinical course of an infant or a child, with a lesion of defined severity, under medical or surgical management. As a consequence of this study, indications for surgery in these three lesions were better defined, the results of surgery in the survivors were critically assessed, and the clinical, non-invasive estimation of severity was sharpened.

Other clinical course studies, based exclusively on Children's Hospital material, published or in press, this past year included investigations of the outlook of patients with Down's Syndrome and congenital heart disease and those with primary myocardial disease. The diagnostic features and the results of surgery in infants with pulmonary atresia and intact ventricular septum, those with interrupted aortic arch, and finally congenital mitral stenosis were presented in separate publications. Our 40 years' experience with bacterial endocarditis has been reviewed and published this past year.

Echocardiography continued to occupy the major portion of Dr. Roberta Williams' research activities. In collaboration with Dr. Charles Tucker, she completed a monograph on cardiac ultrasound with publication expected the end of this cal-

endar year. In addition, in separate undertakings, echocardiographic analyses of left ventricular function after tetralogy of Fallot repair, the nature of left ventricular and right ventricular outflow obstruction in D-transposition of the great arteries, the identification of the morphologic components of congenital mitral stenosis were undertaken.

Dr. Macdonald Dick, in collaboration with Dr. Walter J. Gamble and Dr. John Owens, has been studying the electrophysiologic properties of the conduction system in two clinical settings. In the catheterization laboratory, by means of an electrode catheter, a variety of patient with congenital and acquired conduction disturbances have been investigated. This is of particular importance to define precise indications for pacemaker insertion in asymptomatic children with heart block. A long review article on the use of

pacemakers in infants and children, based on our own material and an extensive review of the literature, has been completed by Dr. Owens and Dr. Gamble for a volume on *Arrhythmias in Children* by Gelband. In the operating room electrical mapping of the conduction system, in the exposed heart, yielded important information on the course of the His bundle in a variety of complex malformations. Corrective surgery in some of these patients, before introduction of mapping techniques, carried a high risk of causing third degree heart block. With the use of this new technique, not only were interesting new observations made, for instance, on the course of the His bundle in L-transposition of the great arteries, but also normal conduction could be preserved in a high percentage of operations.

The increasingly close collaboration with our surgical colleagues resulted in a number of publications redefining indications for surgery and evaluating the results of operative interventions, particularly in young infants. The institutional policy, emerging from these critical studies, at the present time is one of complete correction of severe defects in early infancy, under deep hypothermic cardiac arrest. Palliative cardiac surgery has been virtually abandoned in our institution and the effects of publications of our data, and their presentation at national meetings, strongly influenced the establishment of their principles on the national scene.

A series of papers by Drs. Greenwood and Rosenthal described a variety of *non-cardiac malformations* associated with congenital heart disease. These two investigators are in the process of producing an atlas of their observations, which should prove to be of great assistance both to pediatricians and cardiologists.

II. Myocardial Performance, Metabolism and Ultrastructure

We have had a long-standing interest in the regional oxygenation of the myocardium and distribution of blood flow both in the isolated heart and the open chest dog. The results of these studies indicate that there is a great deal of variation in the oxygen saturation of microscopic cardiac veins; within the same myocardial region both high and low flow situations may exist. The intriguing hypothesis is proposed that increasing afterloads may be met with higher systolic pressures by means of recruitment of a large percentage of the myocardium.

Cardiac hypertrophy has been created in rats both by increased flow and increased pressure loads. Oxygen consumption has been correlated with various performance parameters (developed pressure, developed stress and $\frac{dP}{dt}$) and the reversibility of hypertrophy, after removal of the overload, is being studied.

The coronary vasodilatation abilities of Substance P (a hypothalamic polypeptide, newly synthesized) appears to be different from that of any other substance we have studied in that it does not interfere with local autoregulation. In doses as low as 2×10^{-10} moles/min coronary flow is initially increased by 50%.

Dr. John Owens, one of our trainees, set up a system to study the relationship of ventricular threshold to ectopic beat stimulation (by anodal pulses) and the interval from the preestablished beat (strength-interval curves). The preparation has been stabilized from his previous experience and is being applied to open chest dogs as well as to the isolated heart. A close scrutiny of the strength-interval curves permits prediction of the type of arrhythmias expected from a continuous train of pulses, i.e., whether the heart will fibrillate or have a stable bigeminy.

Finally, studies on the effects of anti-arrhythmic and depressant drugs on the strength-interval curve, as well as arrhythmias, have been started.

Dr. Marlene Rabinovitch, in collaboration with Dr. Walter Gamble and Dr. Lynne Reid, our new Professor of Pathology, is studying the development of pulmonary vascular obstructive disease and right ventricular hypertrophy in rats kept chronically under hypoxic conditions.

III. Pulmonary Physiology in Congenital Heart Disease

Pulmonary function after repair of congenital heart disease is being studied within the framework of this project. A variety of restrictive and obstructive abnormalities have been described and are being correlated presently with the age of the patient at operation as well as the severity and nature of the congenital malformation.

Exercise studies on patients with post-operative tetralogy of Fallot, as well as the general topic of the control of breathing on exercise, are the other major interests of this project.

IV. Physiologic Consequences of Chronic Hypoxemia

In the experimental laboratory, the mechanism of thrombocytopenia, secondary to chronic hypoxia, is being studied in dogs with surgically created right-to-left shunts (pulmonary artery to left atrium). Results, so far, indicate that platelet survival is diminished in these animals; transfusion of these platelets to normal dogs does not prolong their survival time. Through experiments presently underway the effects of eliminating the right-to-left shunt are being investigated. Experiments are also planned measuring blood flow (utilizing microspheres and iron tracers) to bone marrow in rats.

The effects of chronic hypoxemia on physical growth and psychologic as well

as motor development are being investigated. The results indicate that without much doubt cyanosis adversely affects both somatic and intellectual development. Successful surgery, in a group of infants and young children with transposition of the great arteries, resulted in significant increase in weight within the first postoperative year; further increases beyond one year seem relatively unlikely. A study of changes in intellectual and psychological sphere, postoperatively, is now underway.

Current studies in patients with cystic fibrosis are aimed at defining the erythropoietic and hemoglobin oxygen equilibrium response to the hypoxia associated with progressive pulmonary disease.

The cardio-toxic effects of adriamycin in patients with malignancy and the effectiveness of chelation therapy in patients with transfusion hemosiderosis (thalassemia) is being investigated by serial non-invasive studies

V. Cardiovascular Pharmacology

Considerable progress has been made in pharmacologic studies in conscious animals in two categories: A) descriptive effects of pharmacologic agents, and B) use of pharmacologic agents as tools to study reflex regulation of the circulation. The importance of studying these agents in conscious animals has recently been reviewed.

A. Descriptive Effects of Pharmacologic Agents. The effects of morphine sulfate were examined and found to constrict the coronary circulation. Prostaglandin synthetase inhibitors were found to be of importance in the regulation of the renal circulation in response to stress. The role of arterial baroreceptors in mediating the response to a cardiac glycoside was found to be important for cardiac output and heart rate responses, but of little importance for inotropic responses to

digitalis. The effects of three vasoconstrictors, methoxamine, angiotensin II, and vasopressin were compared and found to exert differential patterns of regional vasoconstriction. The effects of isoproterenol were examined on regional myocardial function in the presence of myocardial ischemia.

B. Pharmacologic Agents as Tools to Study Reflex Control of the Circulation. Methoxamine was injected as a bolus to raise pressure and examine reflex cardiac slowing in conscious dogs during severe exercise and after volume loading. In both of these situations reflex cardiac slowing was depressed, indicating that the arterial baroreceptors were turned off during exercise and volume loading. Nicotine was injected proximal to the carotid sinus in conscious dogs to stimulate the carotid chemoreceptors; in that study a new aspect of reflex control of the coronary circulation was observed involving pulmonary stretch receptors. Intravenous injections of norepinephrine and angiotensin II evoked reflex dilatation of the limb, which was mediated by withdrawal of alpha adrenergic tone.

VI. Fetal and Neonatal Cardiovascular Physiology

A. Fetal Peripheral Vascular Responses to Hypoxia. The manner in which fetal peripheral beds respond to hypoxia is being studied in instrumented fetuses in utero. Instrumentation includes Doppler ultrasonic blood flow probes on the renal and iliac arteries and a catheter in the aorta to measure pressure. Hypoxia is induced in the ewe and fetus by administering 10% oxygen to the ewe, resulting in a fall in pO_2 from 23 to 10 mmHg. The fetus responds and these responses are most likely initiated by chemoreflex stimulation. In contrast, the response of the renal bed is biphasic. Initially renal flow rises. After 20 minutes of hypoxia this

response is superseded by a fall in renal flow and renal vasoconstriction. The initial phase was blocked by meclofenamate, an inhibitor of prostaglandin synthesis. Thus, renal flow in the fetus is preserved preferentially in response to hypoxia; the initial renal vasodilatation response appears to be due to a prostaglandin mechanism, and is sufficiently powerful to offset neurally mediated vasoconstriction.

B. Paradoxical Depression of Both Vasoconstriction and of Baroreceptor Reflex Sensitivity in Conscious, Neonatal Lambs. The response of the newborn to I.V. vasoconstrictors (methoxamine, norepinephrine, angiotensin) is being evaluated by comparing effects on cardiac output (electromagnetic flow probes), total peripheral resistance, arterial pressure and on the baroreceptor reflex. Responsiveness to vasoconstrictors depends upon several factors including the extent to which baroreflexes buffer the arterial pressure rise. If baroreflexes are not fully developed in the newborn, then the rise in arterial pressure and sensitivity is assessed by examining the slope of the regression line for the rise in systolic arterial pressure versus the pulse interval. Methoxamine, 100 $\mu\text{g/kg}$, increased mean arterial pressure $18 \pm \text{mmHg}$ and total peripheral resistance $75 \pm 8\%$, while producing a pulse interval/systolic arterial pressure slope of 12 msec/mmHg in conscious lambs 1-2 weeks old. In adult sheep the pulse interval/systolic arterial pressure slope in response to methoxamine was much steeper, i.e., 46 msec/mmHg, indicating a more sensitive baroreflex, while methoxamine increased mean arterial pressure $42 \pm 5 \text{ mmHg}$ and total peripheral resistance $148 \pm 9\%$. Attenuation of the pressor and vasoconstrictor responses in the newborn is also observed following angiotensin and

norepinephrine. Thus, vasoconstrictors elicit a paradoxically blunted pressor response in conscious neonatal lambs, i.e., the depressed baroreflex sensitivity which is observed should have resulted in exaggerated responses for arterial pressure and total peripheral resistance, but actually is associated with striking attenuation of these effects.

VII. Pathophysiologic Correlations and Morphogenesis in Congenital Heart Disease

This is a morphologic-embryologic project consisting principally of the description of a large number of pathologic specimens of congenital heart disease and elucidating their embryologic origins. Of utmost importance, from the diagnostic point of view, is the correlation of morphology with angiographic and echocardiographic appearances.

More recent studies include studies on the coronary anatomy in patients with tetralogy of Fallot. In 4% of the patients the anterior descending branch originated from the right coronary artery; this results in the presence of a so-called Conus Coronary, of considerable surgical importance.

A description of a half a dozen unusual varieties resulted from a detailed study of 93 autopsied cases of total anomalous venous return.

A new classification of transposition complexes was proposed on the basis of 243 autopsied cases from our Cardiac Registry.

A review of our clinical and autopsy material on large numbers of patients with complete truncus arteriosus and with pulmonary atresia and intact ventricular septum, resulted in the description of new, surgically significant, and angiographically definable morphologic characteristics.

VIII. Determination of the Incidence

and Variety of Chromosome Abnormalities in Patients with Congenital Heart Disease

The principal effort for the past year under the direction of Dr. Park Gerald has been directed toward hybrid cell studies of human chromosome 19 as a means of determining the genetic basis of susceptibility to viruses. It is hoped that these studies will lead ultimately to understanding the differences that exist between individuals and between age groups in susceptibility to picornavirus infections, especially to coxsackie viruses. A thorough study of polyethylene glycol induced cell fusion has been completed and a simple, effective procedure for fusing cells in suspension is now available.

During the coming year, the studies of chromosome 19 will be continued, with particular emphasis on the regional assignment of Peptidase D. An attempt will be made to develop a selective system forcing survival of human-rodent hybrid cells to depend upon human Peptidase D. This would permit the production of hybrids carrying various portions of chromosome 19, which hybrids could then be assayed for the coexistence of human receptors for specific enteroviruses.

In addition, we plan to initiate a study of Kartagener's syndrome (the recessively inherited triad of situs inversus, dextrocardia, bronchiectasis and sinusitis). Males with this syndrome have recently been found to be sterile because their sperm are immotile. Upon electron microscopy, the sperm tails have been shown to lack the electron microscopic feature (the "outer arms") believed to be essential for movement. Since respiratory cilia resemble sperm tails in their electron microscopic structure, it is possible that cilia will have similar morphologic lesions in this disease. If so, examination of respiratory cilia may serve as a means of

diagnosing this disease before onset of the respiratory disease (or the infertility). This approach may lead to a better understanding of the genetic heterogeneity of situs inversus and of dextrocardia.

IX. New England Regional Infant Cardiac Program

The New England Regional Infant Cardiac Program, established by a grant from the Maternal Child Health Services of HEW, 1968, represents an attempt to coordinate and improve the care of infants critically ill with congenital heart disease in the six New England states. The effort has been highly successful and resulted in a unique program of nationwide significance. The state of Florida is in the process of establishing a similar program and centers in New York City expressed considerable interest in using this as a model to solve their considerable problems in this area.

The tools are simple. We improved professional education, furnished transportation, supplied social services, improved communications, and established a computerized data base.

In the seven years of the program's existence (1) case finding improved dramatically, (2) the age of admission of infants was lowered, and (3) the salvage rate of infants improved significantly.

On the basis of the data collected by the New England Regional Infant Cardiac Program, meaningful incidence data on congenital heart disease have been collected on almost 2500 infants. Mortality statistics, with and without surgery, under a variety of surgical approaches, have been obtained. The frequency and significance of low birth weight and associated non-cardiac anomalies have been established. The results of detailed physical and psychosocial evaluation of the survivors at five and one-half years of age should throw light on the quality of

the end products of our efforts and may influence future management of these children. The first 250 children examined indicate that their growth, at school age,

under management prevailing 5-6 years ago was depressed, but their I.Q. distribution curve was essentially superimposable on the normal. Both the I.Q.'s

and the psychosocial behaviors were adversely affected by cyanosis, congestive failure as well as poor environment.

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Major Research Projects

I. Development of Artificial Heart Valve Substitutes for Use in Infants and Small Children

Artificial heart valves of various designs, suitable for use in adult patients, have been available for over a decade, and their use has been demonstrated not only to extend the life span of patients requiring valve substitution but also to afford considerable symptomatic improvement. In the case of the infant or child requiring valve replacement, the selection of the valve substitute provides no special problem if the tissue annulus happens to be large enough to accommodate an adult size artificial heart valve. This is not uncommonly encountered as a result of secondary enlargement of the heart and annulus due to the underlying disease process. It is apparent that when an

adult-size valve is utilized hemodynamic performance and clinical benefit are comparable to that found in the adult population requiring valve replacement. However, with decreased annulus size, which is most prominent during the first few years of life, it becomes necessary to specifically define the hemodynamic requirements of the patient and to make some predictions concerning the rate of growth for a given child before selecting the valve substitute which will assure an acceptable period of functional improvement.

For most types of artificial heart valves available, the smaller the tissue annulus, the more borderline the hydraulic function becomes. During the first few years of life the problem is particularly accentuated and, further, the artificial valves currently available for use in patients are either too large or too bulky to provide an

ideal or even adequate substitute. As a result, it is not unusual for cardiologists caring for infants and very young children with serious valve malfunctions to choose an approach of conservative medical management for as long a time as possible. In many instances this results in irreversible impairment of myocardial function or death before the patient can come to the attention of the cardiovascular surgeon. Thus, the need for further efforts directed toward the development of improved heart valves for use in children becomes apparent and forms the subject for the major research endeavor in our laboratory.

Two approaches are currently being explored for the fabrication of the small valves. The first uses glutaraldehyde preserved aortic valves obtained from primate donors (both human and monkey) and mounted on a miniaturized propy-

lene stent. In the other method, valves fashioned from human dura mater preserved in 98% glycerine and mounted on fabric-covered titanium using the technique of Puig, Zerbini, *et al.* is being employed.

In both instances, because the fabric covering used on the stent as well as the sewing ring has to be reduced to the smallest possible dimensions to allow for optimum hydraulic performance for a given sized annulus, emphasis is being placed on maintaining the largest possible orifice diameter to total prosthetic valve size ratio in the construction of each type of substitute.

Experimental studies carried out in our laboratory suggest that it is possible to develop a family of stent-supported tissue valve substitutes suitable for use in tissue annuli of children's hearts in a tissue annulus from 12-22 mm, using either glutaraldehyde preserved stent-supported primate tissue aortic valves or tissue leaflet valves constructed from dura mater preserved in 98% glycerine. In both instances, hemodynamic assessment of the valve substitutes in a mock circulation indicates that function is acceptable at the cardiac outputs normal for infants and children during the first few years of life.

II. Research in Biomaterials: Doubly Tapered Graft for Creation of Systemic-to-Pulmonary Artery Shunts

The concept of improving systemic oxygenation in patients with cyanotic congenital heart disease and diminished pulmonary blood flow and pressure by the creation of a shunt between the subclavian artery and the pulmonary artery (The Blalock-Taussig shunt) was first introduced clinically in 1954. It was found to be particularly effective in the treatment of patients over the age of six months. In younger infants, particularly

those during the first few weeks of life, thrombosis of the shunt occurs frequently. This complication is related in part to the small size of the anastomosed vessels. In an effort to circumvent this problem Waterston, in 1962, created a shunt directly between the ascending aorta and right pulmonary artery. Not uncommonly, some degree of tension is produced on the very small pulmonary artery resulting in late stenosis or occlusion of the pulmonary artery.

Recent advances in the fabrication of synthetic materials has suggested that a doubly tapered graft fabricated of expanded microporous polytetrafluorethylene (PTFE) might provide the basis for an improved method for creating a communication between the aorta and pulmonary artery. This approach has the advantage of allowing for more precise control of the amount of blood flow through the center of the shunt while at the same time providing a tube of larger diameter at each end for performance of the anastomosis.

It is now possible to construct vascular grafts of expanded polytetrafluorethylene having complex configurations. In this series of experiments a double tapered graft is being used to create a bypass shunt between the aorta and the pulmonary artery in rhesus monkeys and between the aorta and the iliac artery in dogs. When the synthetic material is placed under a slight degree of tension, a high degree of patency is achieved in animals sacrificed thus far three to six months following implantation.

The use of this approach in neonates with cyanotic congenital heart disease requiring the creation of systemic-to-pulmonary artery shunts is suggested as a new method to achieve a controlled lumen size and an increased patency rate, while simultaneously decreasing the

incidence of congestive heart failure or of late stricture of the pulmonary artery.

III. Hypothermic Arrest: Nature and Origins of Cerebral Microcirculatory Damage

Deep hypothermic circulatory arrest facilitates repair of complex congenital cardiac anomalies in infants. Susceptibility of central nervous tissue to ischemia, however, remains the major limitation to the technique. Evidence is now accumulating that there is an increase in cerebral vascular resistance after hypothermic arrest. Furthermore, recent investigations have demonstrated the existence of an obstructive lesion of the cerebral microvasculature that increases in severity with increasing time of normothermic circulatory arrest (termed the "no-reflow" phenomenon). We have tested the hypothesis that a microvascular obstructive lesion is closely related to both the elevated cerebral vascular resistance and the brain damage that follows prolonged hypothermic arrest. Further, cerebral metabolic changes have been related to this no-reflow phenomenon.

Combined surface and core cooling with cardiopulmonary bypass has been utilized in these experiments with dogs. Cerebral blood flow is measured by argon desaturation curves and cerebral vascular resistance can then be calculated; cerebral oxygen and glucose consumptions are determined from arteriovenous oxygen and glucose differences. The microcirculation has been evaluated by arterial infusion of carbon black, allowing the unperfused portion of the brain to be quantified. Groups of animals were exposed to various periods of hypothermic arrest and the duration of the arrest related to the severity of the no-reflow lesion and other hemodynamic and metabolic observations.

In a second series of animals, an ap-

appropriate standard time of arrest was chosen and the technique of cardiopulmonary bypass with hypothermia was modified to test the effects of severe hemodilution, dextran, mannitol, and dexamethasone on the development of the no-reflow lesion. Further studies are planned to systematically determine whether the persistent microcirculatory collapse is related to high intracapillary surface tension relative to local reperfusion pressure or, alternatively, to high extra-vascular pressure resulting from cellular or interstitial edema.

IV. Clinical Evaluation of Model X Left Ventricular Assist Device

During the past ten years, we have developed a series of mechanical circulatory support pumps capable of maintaining flows in the range of 4.0-8.0 liters per minute for long periods of time. These studies led to the development of the Model X Left Ventricular Assist Pump, which is a double-valved device that accepts blood from the body of the left ventricle and ejects it into the descending thoracic aorta. Porcine xenograft valves treated with glutaraldehyde solutions are employed in the inflow and outflow sections of the pump, and the entire unit is lined with a unique surface consisting of Dacron fibrils (10 microns in diameter and 300 microns in length) embedded in a polyurethane elastomer. The power source for the device is pneumatic and can be easily synchronized with the R-wave of the electrocardiogram.

Plans have been made this year to implant this temporary left ventricular assist pump in a small series of patients located in five Boston hospitals. A carefully devised clinical protocol was produced to guide patient selection, and this was approved by the Human Resources committees of the five hospitals in the program (Massachusetts General Hospital, Peter Bent Brigham Hospital, Beth Israel Hospital, CHMC, and West Roxbury V.A. Hospital). The results of the first series of clinical pump implantations will be used to guide further developments in this area of mechanical circulatory support.

All of the patients to be selected for pump implantation will be those undergoing a cardiac surgical operation for either acquired, congenital or arteriosclerotic heart disease. The patients' ages will range between 15 and 65 years. Most of the candidates will come from a group of patients who cannot be weaned from cardiopulmonary bypass at the conclusion of the surgical operation. Resuscitation efforts will include pharmacologic support as well as intra-aortic balloon pumping. Failure to respond to these measures would then make the patient a candidate for implantation of a left ventricular assist pump. Technical implantation of the device will be carried out by Dr. Bernhard and his staff. Following pump implantation, a series of physiologic, hematologic and pathology studies are planned for each patient in the series.

V. Development and Evaluation of Unilateral and Bilateral Circulatory Assist Devices

The thrust of this program is further development of an implantable left ventricular bypass device in conjunction with an implantable power source for prolonged circulatory assistance. During the past ten years, we have been engaged in development of a series of left ventricular assist blood pumps which have been totally implantable in calves for periods of up to one year. This particular program involves further development of an electrohydraulic power source, using a transcutaneous technique. Also we will investigate possible improvements in the blood-handling surfaces of the pump, since this is the key factor in the ultimate success of the program. A number of different polyurethane elastomers will be tested to find one which will remain stable without fluid absorption for periods in excess of one year. A number of the polyurethane polymers used in the past have slowly undergone deterioration during chronic calf pump implantations.

Additional studies will be carried out to determine the effects of chronic blood pumping on the platelet survival, red blood cell survival, and fibrinogen turnover. All of these studies are directed toward development of a suitable left ventricular-aortic blood pump for chronic implantation in man.

The Mental Retardation and Human Development Research Program

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Neurologist-in-Chief

Bronson Crothers Professor of Neurology
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Co-Director, Park Gerald, MD
Chief, Division of Clinical Genetics
Professor of Pediatrics, Harvard Medical School

Co-Director, Richard L. Sidman, MD
Chief, Division of Neuroscience
Bullard Professor of Neuropathology
Harvard Medical School

Co-Director, Peter Wolff, MD
Chief, Behavioral Sciences
Professor of Psychiatry, Harvard Medical School

Summary

The overall goal of this program is to provide a setting for research into the cause and pathogenesis of disorders leading to mental retardation and related aspects of human development. The program occupies the first five floors of the Enders Pediatric Laboratories Building and is divided into three major program areas: Genetics, Neurosciences, Behavioral Sciences.

The program approach consists of interdigitation of investigators with clinical background with basic scientists, often with a departmental affiliation with pre-clinical departments at Harvard Medical School such as Microbiology, Pharmacology and Dr. Sidman's appointing department in Neuropathology. Hospital departmental representation consists of

Pediatrics, Neurology, Neurosurgery, Ophthalmology, Psychiatry, and Pathology (Neuropathology). In addition, there is a significant relationship with the Developmental Evaluation Clinic, Director, Dr. Allen Crocker, Associate Professor of Pediatrics, which is primarily a mental retardation training and service unit. The investigative work of the program areas and individual investigators ranges broadly and includes studies of the critical aspects of normal development, as well as the pathological.

Individual projects in various areas of research are outlined in the following section. In addition to the research mission of the Center, there is a rather sizeable effort in research training, in large part individually arranged. There are, however, two formalized research training programs within the Center, one in

Genetics under Dr. Gerald's direction, the other in Developmental Neurology under the direction of Dr. Michael She-lanski, Associate Professor of Neuropathology.

The program, through National Institutes of Health core funding resources, provides administrative support in the various program areas, as well as core-supported laboratories in Histology, Ultrastructure, Electronics, Tissue Culture, Computer Support and specialized support of certain specialized animal facility activities.

The annual budget, derived from the National Institutes of Health, the National Science Foundation and private foundations, amounts to approximately \$3.5 million.

Neurosciences

Chief, Richard L. Sidman, MD

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Edward Sayre, PhD, Suzanne Tarlov, PhD, Ekkhart Trenker, PhD, J.L. Burchfiel, PhD

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Francois Rieger, PhD, Dennis Selkoe, MD, Virginia Lee, PhD, Shu-Hui Yen, PhD, Ronald Liem, PhD, Merle Ruberg, PhD

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Christo Goridis, MD (Visiting Assistant Professor)

Financial Support:

Brain Development in Normal and Mutant Mice, NINCDS, \$192,618, Dr. Sidman.

Growth and Regeneration in the Nervous System, NINCDS, \$225,454, Dr. Sidman.

Neurological Evaluation of Electroencephalogram Records, Edgewood Arsenal, \$15,000, Dr. Duffy.

Pharmacology of the Forebrain, NINCDS, \$76,000, Dr. Barlow.

Foundation for Hereditary Diseases, \$4,000, Dr. Snodgrass.

Drug, Vitamin and Hormone Transport in Spinal Fluid, NINDS, \$36,382, Dr. Spector.

Transport of Gentamicin, Folate and Thyroxine in the Central Nervous System,

National Foundation — March of Dimes, \$22,000, Dr. Spector.

Developmental Neurology, NINCDS, \$469,414, Dr. Barlow.

Genetic Defects of the CNS: Interaction of Mutant and Normal Cells in Experimental Chimeras,

National Foundation — March of Dimes, \$22,000, Dr. Mullen.

Biology and Pathology of the Neuronal Fibrous Proteins, National Science Foundation, \$49,240, Dr. Shelanski.

Developmental Neurology Training Program, NINDS, \$125,550, Dr. Shelanski.

Neurogenetic Processes in Fetal Brain, NINDS, \$108,740, Dr. Rakic.

Biochemical and Genetic Analysis of Steroid Receptors from Normal and Mutant Mouse Brains,

National Foundation — March of Dimes, \$19,500, Dr. Fox.

Characterization of Antigenic Components on Normal, Malignant and Mutant Mammalian Brain Cell Surfaces,

National Foundation — March of Dimes, \$20,000, Dr. Schachner.

Deutsche Forschungsgemeinschaft, \$12,000, Dr. Kunzle.

Studies on Neural Cell Surface Components, NINDS, \$48,969, Dr. Schachner.

Retrograde Axonal Transport in the Visual System, NINDS, \$52,000, Dr. J. LaVail.

Mellon Faculty Award, \$15,000, Dr. J. LaVail.

Neurosciences

(continued)

Research and Education Funds, V.A. Hospital, West Roxbury, Mass., \$34,000, Dr. Kneisley.

Cellular Mechanisms of Inherited Retinal Degeneration, NEI, \$43,646, Dr. M. LaVail.

Research Career Development Award, NEI, Dr. LaVail.

Abnormal Movements and Pharmacology of the Forebrain, NINDS, \$67,864, Dr. Snodgrass.

Mode of Action of the Nerve Growth Factor, National Foundation — March of Dimes, \$20,000, Drs. Greene/Shelanski.

Neurochemical Studies of Cultured Sympathetic Neurons, NINDS, \$18,577, Dr. Greene.

Pathology and Biology of the Neuronal Fibrous Proteins, NINDS, \$49,240, Dr. Shelanski.

Development of the RCS Rat as a Model of Hereditary Degenerative Disease of the Retina, NEI, \$235,639, Dr. Sidman.

Major Research Projects

I. Brain Development in Normal and Mutant Mice.

This project asks, what classes of developmental processes do genes control during formation of the mammalian nervous system, and what epigenetic cell-cell interactions are critical for differentiation and the establishment of synaptic connections? A very relevant byproduct is the elucidation of animal models of developmental diseases that lead in humans to mental retardation and other serious malfunctions of the nervous system.

We focus particularly on inherited diseases affecting development of the cerebellum. Study of the weaver, staggerer, and reeler mutants has indicated that the selective deficit of cerebellar granule cell neurons in these diseases results not from mutant gene action in the granule cell itself, but in a different type of neighboring cell. In weaver, the earlier defect seems to be in a glial cell which normally provides cell surface-mediated directional guidance as the young granule cell neuron becomes transposed from its site

of origin on the surface of the cerebellum to its mature site internally. In staggerer and reeler the basic problem is hypothesized to be with the Purkinje neuron, and the basically healthy granule cell dies because it is unable to form synaptic connections with its neighbor which is abnormal in form (staggerer) or both position and form (reeler).

Whether these cell-cell relationships are coincidental or causal is under test by experimental methods. Messer has developed a monolayer culture method for dissociated early postnatal cerebellar cells, and finds that staggerer and weaver granule cells survive *in vitro* at least as well as cells from wild-type mice. In another culture system, Trenkner and Hatten find that immature granule cells of weaver mice fail to generate processes, but can be induced to do so by chemical modification of the culture medium. Process formation is also modified in these cultures by reagents that block selected cell surface carbohydrates, suggesting involvement of surface glycoproteins or glycolipids in cerebellar cell differentiation. In a separate report, Mullen describes the use of experimental

chimeras to analyze these and other cell interactions that have been postulated on the basis of the morphological studies.

The cerebellar Purkinje cell, unlike the granule cell, develops relatively independently of its local cellular milieu. We have described two new mutants with a relatively selective postnatal degeneration of Purkinje cells, named nervous *nr*, and Purkinje cell degeneration, *pcd*. The completeness and precision of the lesion in *pcd* makes these mice particularly suitable for analysis of synaptic specificity. The possibility of synaptic reorganization was suggested by the intriguing fact that *nr* and *pcd* mice are only slightly uncoordinated despite that massive loss of Purkinje cells, generally considered to be the central elements of the cerebellar circuitry. Our search for an expected synaptic reorganization that would correlate with the preservation of relatively good function has been negative to date; a quantitative study of synapses made by Purkinje cell axons onto cell bodies of deep cerebellar neurons shows that the degenerated axon terminals are not replaced. A further unexplained mystery discovered by Tarlov is that while gamma-aminobutyric

acid (GABA, the neurotransmitter used by Purkinje cells) is moderately reduced in cerebellar deep nuclei of *pcd* mutant mice, activity of the biosynthetic enzyme, glutamic acid decarboxylase remains virtually normal. These studies are continuing as part of a general plan to understand the responses of inhibitory synaptic systems following nervous system injury.

A significant part of our effort goes into the description and analysis of new mutants, each of which presents a unique experimental paradigm with, presumably, as many different molecular mechanisms as there are independent genetic loci involved. Four as yet unpublished cerebellar mutants are under active study, named cerebellar outflow degeneration, vibrator, meander, and the as yet unnamed "neuromuscular 334". An autosomal recessive mutation named shiverer causes a very severe deficit of myelin throughout the brain and spinal cord and repeated epileptic seizures; it appears to be genetically independent of the quaking and jumpy myelin-deficient mutants we had described earlier. Two new, as yet unnamed, mutants show a combination of pigment spotting and severe hydrocephalus.

One technological advance merits brief mention. Sayre and Pearlstein are leading us in developing a computer graphics facility for display and quantitative analysis (surface areas, volumes, etc.) of three-dimensional objects (e.g., individual cells, fiber paths, synaptic patterns) reconstructed from serial sections at the light or electron microscopic level. We have published our first study, an analysis of the shapes, volumes, and surface areas of young neurons migrating to the cerebral cortex of the fetal brain. Linkage of the computer to a light microscope will shortly allow direct tracing and quan-

tification of cell contours and of autoradiograms. This approach, also being developed in several other laboratories, is essential if a quantitative element is to be introduced at last into the morphological analysis of neural development.

II. Studies on Experimental Amblyopia
Hubel and Wiesel developed the experimental model of cat amblyopia, which differs from ordinary human *amblyopia ex anopsia* in being produced by closing an eye in infancy rather than resulting from strabismus. Hubel and Wiesel showed that if eye closure was begun in infancy and continued to adult life, the cat behaved as if nearly blind in the deprived eye, that only slight behavioral recovery occurred even with closure of the good eye, and that such cats appeared to have lost input to visual cortical neurons from the deprived eye. Single unit physiological studies indicated normal responses and receptive fields when the good eye was stimulated, but very few cortical neurons could be driven from the deprived eye, and those which did respond usually had abnormal receptive fields. This finding has been taken as demonstrating an irreversible loss of function and synaptic connections related to the deprived eye. For several reasons relating mainly to studies of humans with amblyopia, we suspected that this theory might be wrong and that active inhibitory suppression might be the principal factor. Because of other experiments suggesting that bicuculline, a known GABA receptor blocker, could alter receptive fields and visual function of normal cats, we gave bicuculline IV to cats with experimental amblyopia who showed the typical loss of input to visual cortical neurons from the deprived eye. We found that about two-thirds of neurons studied showed a rapid restoration of responsiveness to stimulation of

the deprived eye. More important was the finding that receptive fields for the deprived eye after responding to bicuculline were nearly identical to those in the normal eye, indicating that response specificity had not been lost in spite of prolonged suppression of function. An editorial in *Nature* suggested that this response specificity which was retained might derive from the few days of visual experience which our cats did have prior to lid suture. We have sutured some cats prior to eye opening and expect that they will have the same receptive field characteristics in view of the relative independence of most such characteristics from any visual experience.

Although this work was very exciting, bicuculline is a difficult and dangerous drug. It often produces convulsions, its action is very brief (typically 1-3 minutes, sometimes longer) and it can not safely be given to man. We have now found a way to obtain a more prolonged response using ammonium acetate, which acts distal to the receptor, to interfere with active chloride transport, thereby reducing or eliminating synaptic potentials which depend upon changes in chloride permeability. This approach was first used by Lux on synaptic motoneurons and is appealing because the effect lasts 30-90 minutes, long enough to readily allow behavioral testing, the dosage required is not large, and EEGs of our animals suggest that there is much less tendency to provoke seizure discharges than is true of bicuculline. We find that some cells which do not respond to bicuculline do respond to ammonium acetate infusions by restoration of binocularity. One interpretation is that this effect indicates inhibitory suppression by non-GABA neurons, although other explanations are possible. Ammonium infusions appear to alter receptive fields

of the normal eye much more than does bicuculline, again suggesting a possible role for non-GABA inhibitory neurons in receptive field shaping.

We do not know where bicuculline acts to restore binocularity in these cats. We are planning iontophoretic studies of bicuculline and related drugs in both lateral geniculate and visual cortex and feel that these studies will enable us to prove also that the bicuculline effect is related to its ability to block GABA receptors, something which we only assume at present. We are planning also behavioral studies to prove that useful vision is restored in these animals. Because of the good correlation found in the past between single unit physiology and visual behavior, we are optimistic that we shall be able to demonstrate useful vision after drug treatment. We are also planning a long term series of studies using radioactive bicuculline as a receptor label to study the number and spatial distribution of GABA receptors in lateral geniculate and visual cortex and their relationship to visual experience. We know that many of the primary afferent connections in the visual system are formed before birth and can not therefore depend upon experience, and suspect that local neurons may differ in this respect and that inhibition may be at least partially dependent upon visual experience. Because human amblyopia rarely relates to deprivation we are also planning to study experimental strabismus and visual defects related to strabismus. We feel it is particularly important to have the same laboratory studying physiology, pharmacology, chemistry and cell biology of the same phenomena in the same animals, rather than having one laboratory studying physiology and another in another city studying biochemical changes in amblyopic animals, etc.

Drs. Burchfiel and Duffy have completed also a series of studies of the somatosensory system of the rhesus monkey with special emphasis upon cells responding to several sensory modalities. For example, they have identified a class of complex cells in area 5 of the rhesus brain whose response can differentiate between having the hand open and closed and can also indicate whether or not the closed hand contains an object. In another area, 2v, the so-called vestibular area, cells have been studied which respond to auditory, visual, vestibular and somatosensory input (quadrimodal cells) and whose function might relate to protection of the face from objects moving rapidly toward the face. Related studies have also studied the strong corticofugal inhibitory effect on units in the somatosensory thalamus. This potent inhibitory pathway may be analogous to pathways operative in suppressing input, at the geniculate level, to visual cortex of our amblyopic cats.

Drs. Duffy and Burchfiel have also performed a number of studies related to analysis of abnormalities found in electroencephalograms and evoked potentials of humans with single exposures to organophosphorus anti-cholinesterase drugs. Of special interest is that a single exposure appears to produce a definite abnormality which lasts for years, long after cholinesterase levels can be shown to be normal. This effect suggests the possibility that these drugs somehow produce necrosis or destruction of nerve terminals, perhaps in a fashion analogous to 6-hydroxydopamine and we are examining this possibility in experiments with rats. A drug which could selectively destroy cholinergic terminals without killing the rat would be extremely valuable in neuropharmacology. For example, we are especially interested in the func-

tion of local neurons in the visual system. The only way to destroy local neurons without simultaneous destruction of long axon relay neurons is by use of chemical or immunological means and such chemicals would be extremely useful for many purposes.

III. CNS Transmitter Pharmacology

The work of this group centers around transmitter pharmacology, emphasizing mechanisms and regulation of transmitter biosynthesis in several mammalian systems. Dr. Beart has been studying synthesis and release of the amino acid transmitter, glycine, in rat spinal cord *in vitro*, using several different radioactive precursors, because many synthetic pathways for glycine synthesis already are known and it remains unclear which one or ones are responsible for synthesis of that glycine which is used as a transmitter. The main criterion used to demonstrate "transmitter glycine" has been that glycine, which can be released in a relatively specific fashion, is releasable from tissue slices and synaptosomes *in vitro* by a high potassium mechanism which is calcium-dependent (as appears to be true of all known transmitters *in vivo*). Glycine released into the medium in calcium free media is subtracted from the value obtained when calcium is present. Under these conditions, some transmitter glycine appears to be formed from not only serine but also glyoxylate and pyruvate, the latter result requiring a previously unknown synthetic pathway. A similar approach has been used by Drs. Beart and Snodgrass to study synthesis and release of γ -aminobutyric acid (GABA), a major inhibitory transmitter in mammals, from radioactive glucose and acetate. With double label techniques, it can be shown that GABA labelled from acetate is non-releasable and that the released GABA is derived almost entirely

from glucose. This approach may permit a judgment to be made about GABA depletion after brain lesions: for example, knife cuts between striatum and substantia nigra result in a decrease in GABA in the substantia nigra. Double label studies might permit us to decide whether the GABA decrease is a loss in "transmitter GABA" or "metabolic GABA". This approach will also be used in studies of the development of GABA synthesis in specific brain areas and its relationship to innervation and environmental factors.

We have used the technique of drug injection directly into the substantia nigra of unanesthetized animals to study the pharmacology of drugs which inhibit glutamic acid decarboxylase (GAD), the enzyme responsible for GABA synthesis, as well as gaining information about the interrelationships between GABA releasing nerve terminals in the substantia nigra and the nigro-striatal dopaminergic neurons, which seem to be important in several types of motor behavior. To date, only allyl glycine has been studied in detail, but further studies will employ other GAD inhibitors as well.

Drs. Uretsky and Snodgrass have studied the detailed regulation of the activity of the enzyme tyrosine hydroxylase, which is rate-limiting for catecholamine biosynthesis in both peripheral and central nervous tissue. Increased firing of catecholamine neurons, or depolarization by high potassium media, as described above for glycine, have been known to result in increased activity of tyrosine hydroxylase (TOH). It has been assumed that this increase reflects a release of feedback inhibition of the enzyme, because it is known that catecholamines do inhibit TOH. As amines are discharged from nerve endings, their levels would decrease in a hypothetical pool, resulting in reduction of inhibitory effects upon

TOH. Our work has shown that drugs such as amphetamine and tyramine can stimulate TOH even after blockade of amine synthesis at the decarboxylation step. When rats are given two doses of reserpine to deplete stored catecholamines, and slices of their striatum incubated with a decarboxylase inhibitor, no releasable dopamine remains. However, it is still possible to markedly increase TOH activity in the slices with amphetamine and similar drugs.

These results suggest another form of short-term TOH regulation in addition to feedback inhibition, whose existence is not in dispute. Preliminary studies with various ionophores and ion-free media suggest that this new form of regulation involves entry of extra-cellular calcium and is independent of changes in cyclic nucleotides. Our studies on the ionic bases of this regulation are continuing and we are planning to explore the phenomenon in homogeneous cell lines in tissue culture (such as Dr. Greene's pheochromocytoma line) if these cells show regulation. These studies also provide another basis for categorizing stimulant drugs and exploring their mechanism of action which seems to involve more than simply releasing endogenous transmitter.

Another project involves the biochemical and pharmacological study of a hereditary motor disease, strio-cerebellar degeneration of the Kerry Blue Terrier. This disease was first described in 1946 but was not recognized as hereditarily determined until recently. Dr. Averill of our institution first drew our attention to this very interesting disease and has been involved in liaison with dog breeders, clinical evaluation of the dogs, and histopathological studies. Our interest has been to understand the motor phenomena, chorea, and ataxia, in terms of ab-

normalities in transmitters in the brain and to attempt therapy with transmitter-related drugs. The disease is inherent as an autosomal recessive and characterized by onset of tremor and chorea in the 2nd-4th months of life, with progression to severe ataxia, inability to walk, eventually to sit and death from inanition. Systemic abnormalities have not been recognized in any of these dogs, and their brains show several interesting lesions, including extensive loss of neurons in the pars compacta of the substantia nigra, necrosis of the caudate, lesions of inferior olive, cerebellar cortex, and loss of Purkinje cells. In spite of extensive loss of neurons in the substantia nigra, the dogs never show any akinesia or Parkinsonian features, presumably because of the effect of co-existing brain lesions. Biochemically, we find what seems to be marked decreases in TOH and GAD in several regions, including caudate and substantia nigra, although we must have more biochemical values on control dogs before we can be sure that the affected dogs do in fact have a decrease in transmitters in certain brain regions. The fact that more than one transmitter system appears to be abnormal, and not in all brain regions suggests that the transmitter abnormality is secondary rather than primary and in this respect is similar to Huntington's chorea, where at least two transmitter systems appear to be affected, but only in a few brain regions. This disease is more similar to olivo-ponto-cerebellar atrophy than Huntington's disease, but is a valuable model system to study in detail. Our interest is increased by the fact that at a moderately advanced stage of the disease, the dogs are unable to walk and then can walk after treatment with haloperidol. However, with further progression, haloperidol becomes ineffective. This might be explained in terms

of a stage where there is increased sensitivity (whether due to receptor changes, loss of uptake mechanisms, etc. to dopamine) followed later by either loss of ability to make dopamine or total disappearance of receptors in the area where increased sensitivity was present. The progression through several stages with loss of responsiveness to a therapeutic agent resembles the progression shown by several human diseases, such as Parkinsonism. The cause of this progression and alteration in drug responsiveness is unknown in the human diseases and this animal model should be useful. We expect to continue working with this model disease for several years, since only a few of the obvious studies have been done so far.

Other work includes study of the effects of local injections of drugs into the raphe nuclei of the rat brain stem, where all the cell bodies of the serotonin neurons of the brain are believed to be. We are using drugs which are relatively selective in destroying serotonin neurons without effect on nearby norepinephrine neurons and then studying turn-over of dopamine in striatum, cortex, and other regions. We observe that asymmetrical raphe lesions produced by drugs such as p-chloroamphetamine produce turning which is blocked by neuroleptics, suggesting that it is dopamine-mediated. We hope to be able to explain this motor behavior in terms of an asymmetrical release of forebrain dopamine systems from inhibition by serotonin neurons. These studies require the simultaneous measurement of both serotonin and catecholamine turnover in individual brain regions after administration of a decarboxylase inhibitor.

IV. Central Nervous System Transport

In the last 18 months, we have studied the transport and regulation of vitamins,

drugs, hormones, and carbohydrates between blood, cerebrospinal fluid (CSF) and brain. Our special focus has been on the transport systems in the choroid plexus and the relationship between homeostasis and transport.

Vitamins: The vitamins ascorbic acid, thiamine, folates and inositol have been studied in the central nervous system. We have found certain general principles about these vitamins. First, the level of each vitamin is higher in CSF than plasma. Secondly, the entry of each vitamin from plasma into CSF and brain is saturable with the one-half saturation value approximately equal to the blood value. Since these vitamins are not synthesized in brain, the specific saturable transport systems for these vitamins in brain allow regulation of the vitamin levels in CSF and brain. This is accomplished by replacing the vitamins which constantly turn over (as by bulk flow of CSF). In summary, then, these transport systems serve as regulatory or homeostatic systems.

Further investigations have revealed that the locus of these transport systems is, in part, the choroid plexus, the anatomical barrier between plasma and CSF. At present, we have isolated and are characterizing the folate binding protein that is involved in the transfer of folates between blood and CSF.

Drugs: In the last 18 months, we have studied in detail why the drug, gentamicin, does not enter CSF in adequate amounts. The results show that the predominant reason is that there is poor entry of gentamicin into CSF.

Hormones: A study of the hormone, thyroxine, revealed it to be taken up by a saturable mechanism in the choroid plexus. This system was consistent with facilitated diffusion and probably explains the rapid, saturable entry of

thyroxine into CSF.

Carbohydrates: Glucose has been thought to be actively transported by choroid plexus. However, a careful study of this question shows glucose probably enters choroid plexus (and CSF) by facilitated diffusion.

V. Gene Action and Cell Interactions in Experimental Chimeras

Chimeric mice are made by the *in vitro* aggregation of 8-cell embryos from one strain with those from another strain. After culturing overnight, the double-sized embryos are surgically transplanted to the uteri of host females where normal development ensues. The tissues of the resulting chimeras are mosaic, containing mixtures of cells of the two parental genotypes. Our objectives in studying central nervous system mosaics are 1) to determine in which cell type(s) particular mutant genes are acting, 2) to see how coexisting mutant and normal cells interact, and 3) to analyze mosaic patterns for evidence of clonal development and/or cell intermingling and migration.

Our first look at mosaicism in the brain followed the discovery of a new mouse mutant, Purkinje cell degeneration (*pcd*), which we have recently described. In homozygous mutants, all cerebellar Purkinje cells degenerate postnatally. We used *pcd* to make mutant-normal chimeras (i.e., *pcd/pcd* - +/+) and were able to see mosaicism in the brain for the first time, for in the chimeras many but not all of the Purkinje cells had degenerated. By using beta-glucuronidase (a separate mutation) as an independent histochemical cell marker we found that all of the surviving Purkinje cells were, in fact, genetically normal cells indicating that the *pcd* gene acts within the Purkinje cell. Preliminary serial reconstructions have, surprisingly, failed to reveal any clonal pattern, suggesting there may be more

cell mixing in the developing CNS than previously thought.

In a reeler (*rl*) chimera, patches of abnormally positioned Purkinje cells, a characteristic of reeler mutants, were found. These patches contained both genetically reeler and genetically normal cells suggesting the cells are being positioned by factors extrinsic to the affected cell type. In staggerer mutants the cerebellar granule cells degenerate but the Purkinje cells are also abnormal. With *sg/sg* - *+/+* chimeras, Dr. Herrup has found that the gene that causes the granule cells to degenerate is acting in the Purkinje cell.

To study inherited retinal dystrophy in the rat, we produced *rat* chimeras and found that, although it is the photoreceptor cell that degenerates, the mutant gene is actually acting within the pigment epithelial cell (see also M.M. LaVail).

As an outgrowth of our work on glucuronidase histochemistry we have found direct evidence for intercellular exchange of this enzyme under normal physiological conditions. This finding is of broader interest because of its relevance to diseases resulting from enzyme deficiencies.

VI. Studies on Cellular Fibrous Proteins

The primary interest of the laboratory centers on the role of the cellular fibrous proteins in the control of cell form, its genesis and its mechanism. As related problems came up in the work such as the components of the cell surface during differentiation and the mobility of cell surface components, we have carried out projects of a somewhat secondary nature as well. During the past year our work on microtubules has been greatly attenuated, though we have continued to study the role of both calcium and GTP on microtubule assembly and disassembly *in vitro*. These studies have led us to question the commonly held assumption

that calcium is the primary regulator of microtubule assembly. The fact that glycerol, which can be viewed as a "water structure", inhibits the calcium effects and that tubules assembled with GMPPCP will not disassemble seem to argue for a much more subtle and complex regulatory process.

Work on the intermediate filaments (100A filaments) has continued. The protein has been highly purified from brain and antibodies prepared against it. A combination of biochemical and immunological approaches have shown the protein to differ from actin and tubulin, though some peptide map similarities with tubulin suggest a common evolutionary ancestor. In the brain the antibody has been localized to both neuronal and glial filaments, as well as the axonal filaments of peripheral nerve, using horseradish peroxidase coupled antibodies for EM immunocytological localization. A somewhat surprising result was that the antibody also localized to the postsynaptic densities. While we were initially skeptical about this, direct immunological studies on purified densities shows them to be identical to the filament on immunological and electrophoretic grounds. The intermediate filaments are not limited to brain. Our immunocytological studies (together with Drs. Blose and Chacko, U. Penna. Vet. School) show clearly that the perinuclear ring of 100A filaments in the rodent aortic endothelial cells are composed of the same antigen and that this immunoreactivity redistributes in the same way as the filaments after colchicine treatment. The presumptive 100A filament protein from smooth muscle cells also shows immunological and electrophoretic similarity, if not identity, to the brain filaments. Therefore, we are quite convinced that the intermediate fil-

aments represent a highly conserved and broadly distributed class of fibrous structures which, on the basis of their extreme stability, are likely to make up the primary cellular cytoskeleton. Further developmental and biochemical studies are to be directed to the investigation of this hypothesis.

In collaboration with Dr. Lloyd Greene, we have investigated the molecular isomers of acetylcholinesterase (AChE). Using culture cells and a combination of reversible cholinesterase inhibitors that do not pass the cell membranes and DFP, an irreversible inhibitor which enters the cell readily, it has been possible to show that the 4S form of AChE is confined to the cell interior while the 10S form is limited to the plasma membrane. Detailed studies on the induction of these forms and their control in a variety of cell types are underway.

A number of neuropathological studies on the nature of the neurofibrillary degenerations in such conditions as infantile neuroaxonal dystrophy, mongolism and Alzheimer's disease continue with the weight of the evidence indicating that these abnormal filamentous structures are related to, and possibly derived from, the normally occurring neurofilaments.

A new area of research aimed at "mapping" the proteins of the cerebella of normal and mutant mice has been started. The staggerer mutant which has been the mutant most extensively studied to date shows the appearance of a new protein band in the particulate fraction between 7 and 14 days. This band is immunologically identical to the intermediate filament protein and is therefore probably a reflection of the intensive secondary gliosis which occurs in these animals at this period. Current studies are aimed at determination of whether the anti-filament antibody can inhibit the for-

mation of gliosis. Were this to be the case it would have important implications for neural regeneration.

VII. Neurogenetic Processes in Primate Brain

The primary goal of the section concerned with Primate Brain Development is to elucidate basic principles and mechanisms involved in the genesis of the central nervous system in nonhuman primates in order to ultimately understand the organization of the brain in patients afflicted with developmental diseases, including salient abnormalities which may lead to mental retardation. In addition, the analysis of the axonal growth, neuronal connectivity and synaptogenesis during genesis of the normal primate brain may provide a sufficient understanding of regenerative cell behavior to make an attempt to promote reestablishment of functional connections between neurons after damage to the human nervous system. Experimental analysis of the cellular developmental events directly in monkey brain proved to be an essential step in the pursuit of our goals because our previous studies on the neurogenesis in man showed that certain brain regions, particularly those which reach their peak in size and complexity in primates develop according to principles not utilized for the construction of less complex rodent brains. In addition, we have found that protracted time span necessary for the development of a large gyrencephalic brain and the enormous number of generated neurons require somewhat different developmental mechanisms and enable one to formulate some fundamental questions that are not possible to answer by the analysis of a smaller, fast growing laboratory animal.

To analyze rationally the process of neurogenesis in primates, a systematic series of monkey brain specimens have

been built by Dr. Rakic that allow combined autoradiographic, Golgi and electromicroscopic analysis of development in the number of selected brain regions. Fetuses and early postnatal animals were exposed once to H^3 -thymidine and were killed hours to many months later by perfusion of the circulatory system with a buffered formaldehyde-glutaraldehyde mixture. Blocks from one half the brain were processed for autoradiographic analysis, while small samples from the other side were postsmicated and embedded in plastic for electronmicroscopy and the remainder of that half impregnated by a variant of the rapid Golgi technique and sectioned serially. In addition, we have recently developed a new method for correlating the time of a neuron's origin with its ultimate projection by combining (H^3)-thymidine autoradiographic and horseradish peroxidase histochemical procedures. By this new method, it is now possible for the first time to identify an additional essential property of (H^3)-thymidine labeled neurons other than their appearance or position in a given brain structure.

During the past year another important, completely new approach has been developed which allows analysis of connectivity and synaptic plasticity in the developing primate brain. We refined the surgical procedure by which the nervous system of the rhesus monkey can be manipulated intra-utero from the 50th day of pregnancy to birth, preserving the fetus which is subsequently allowed to survive for various intervals following surgery, including birth of such fetus through normal delivery and effective postnatal survival. This procedure allows analysis of neuronal connections before birth using autoradiographic methods of retrograde and anterograde axoplasmic transport as well as transneuronal trans-

port in fetus killed 14 days after injections of radioactive amino acids and sugars into various brain regions. In addition, intrauterine surgery provides the unique possibility for experimental analysis of the effects of selected lesions afflicted on the fetal primate brain. In the first several successfully operated monkey fetuses, one or both eyes were removed at different stages of pregnancy, animals delivered, sacrificed at 2-3 postnatal months and processed for analysis.

By now well established multi-prong experimental attack, selected complex primate brain regions have been analyzed in detail and several new ideas on the dynamics of primate brain development have emerged. These can be found in our published records in the last several years. The studies presently conducted concern: neurogenesis of the visual and frontal cortex; the time of neuron origin and synaptogenesis of the lateral geniculate body and histogenesis of the hippocampus. Our analysis has demonstrated that stages of differentiation and onset of synaptogenesis in primates differ from that of non-primate species not only with respect to the time of birth, but also in the relative duration of each stage of maturation and in the total time necessary for achieving a given level of neuronal differentiation. The timetable of cell genesis, synaptic formation and other parameters of brain development in rhesus monkey have been correlated to the data available for the human neurogenesis, which makes experimental results in laboratory animals more relevant to an understanding of the pathogenesis of certain developmental brain disease in man.

In the past year our study of prenatal development of visual connections by autoradiography in fetuses sacrificed 14 days after unilateral eye injection with a

a mixture of radioactive proline and fucose showed that initially retinotectal and retinogeniculate projection from both eyes overlap. Segregation of the input to the superior colliculus and lateral geniculate body from the two eyes occurs during the middle of gestation. Transneuronal transport of radioisotopes indicate that the optic radiation is formed in the first half of gestation by fibers from the lateral geniculate body but these fibers do not enter the developing cortical plate before their target neurons reach the IVth layer of the primary visual cortex. These results show that the organization of the visual system in the adult primate is achieved through several transient steps that occur before birth. Presently we are analyzing the extent of re-arrangement of axon terminals that has been demonstrated in recent experiments in which components of the visual system have been destroyed by intrauterine neurosurgery on fetal brain at critical periods in primate gestation.

VIII. Biochemical and Genetic Analysis of Sex Steroid Binding Macromolecules in Developing Mouse Brain

Both genetic and hormonal factors have been demonstrated to influence sexual differentiation in newborn rats and mice. By combining the genetic analyses that are possible using inbred strains and mutants of mice, with affinity methods for fractionating steroid receptors, we have demonstrated, analyzed and compared three sex steroid binding activities in developing mouse brain. These studies provide a biochemical system for asking how genetic and environmental determinants interact in this particular developmental model.

Previously, investigators had demonstrated the presence of estradiol receptors in several regions of rat brain including hypothalamus. We provided the first

direct evidence for the presence of adult-like estradiol receptor in neonatal mice by using the affinity method of DNA-cellulose chromatography.

With 2-day-old mice we separated estradiol receptor and "neonatal binding protein" from extracts of hypothalamus plus preoptic area. The receptor has high affinity for estradiol, diethylstilbestrol and DNA, while "neonatal binding protein" has lower affinity but a higher capacity for estradiol and interacts very little with diethylstilbestrol and DNA. These experiments support the concept that "neonatal binding protein" protects neonatal brain from high maternal estrogen levels that might otherwise "estrogenize" all young mice and rats, causing them to be sterile.

Our studies of the estradiol receptor suggest that its binding to DNA and its conversion to the "nuclear" form differ for brain and uterus. That these differences may be physiologically meaningful is suggested by three recent *in vivo* findings that parallel our cell-free observations closely. Thus we are pursuing a comparative study of the mechanisms of estradiol receptor behavior and interconversions.

In tissues from rat brain, androgen binding proteins similar to the estradiol receptor have been reported by some investigators, while others have challenged the specificity and significance of these findings. We have found high affinity androgen binding in brains of mice as young as 2 days old. These binding components are missing (95% reduced) in extracts from the androgen-insensitive mutant mouse, testicular feminization (Tfm), supporting the conclusion that they are genetically and physiologically significant.

These studies of androgen and estrogen binding in mouse brain led to the

hypothesis that the receptor mechanism for sex steroids detects the *ratios* of estrogens to androgens, rather than independently detecting absolute levels of each type of hormone. In support of this hypothesis, we have found that estradiol can block all detectable androgen binding at concentrations in the range of the androgen affinity constant. Thus the estradiol receptor binds estradiol alone, while the androgen binding component has affinity for both androgen and estrogen. These studies are being pursued in both brain and uterus, where the same two components are found, and may lead to new concepts of sexual differentiation and behavioral response based on a biochemical mechanism for detecting hormone "balance".

Estrogens and androgens have also been shown to affect cerebellum. The tools used above, along with neurological mouse mutants, were used to establish the specific affinity of macromolecules from mouse cerebellum for both estrogens and androgens. This finding is important because other studies with rat brain extracts have indicated lower levels of steroid binding in cerebellum compared to regions such as hypothalamus and preoptic area, but without evidence that these lower binding components are specific. Our studies will now allow investigations of the mechanisms of sex steroid functions in cerebellar development and function.

IX. Immunological Analysis of Cell Surface Components in Adult and Developing Mouse Brains

It is generally believed that cell surface components play a crucial role in cell-cell interactions during morphogenesis and differentiation of the nervous system. We have started the identification and characterization of nervous-system specific cell surface components by using im-

munological techniques. The serological characterization of nervous-system specific antigens has developed in several directions: (1) Biochemical identification of antigens, (2) Immunohistological localization of antigens, (3) Definition of new nervous-system specific cell surface antigens, and (4) Immunoselection of antigen-positive and -negative cell populations.

A. Biochemical Identification of Antigens. NS-2 has been partially characterized by cell surface radioiodination and immunoprecipitation as an antigen carrying a glycolipid component(s) and a peptide with a molecular weight of approximately 50,000. Glioblastoma, glioma 26 and brain which had previously been found antigen-positive by the cytotoxicity test contained these compounds, whereas thymocytes, spleen, sperm and C1300 neuroblastoma which had been negative in cytotoxicity tests were also negative by the more sensitive radioimmunoassay method.

B. Immunohistological Localization of Antigens. Antisera to NS-2, NS-3 and NS-4 have been applied to 10 μ m sections of cerebellum from 21 day old mice and visualized with the indirect immunoperoxidase method. NS-4 was found to be highly positive throughout the molecular layer and in the glomeruli of the granular layer, whereas the outlines of the granule cell bodies were less intensely stained. NS-2 and NS-3 showed some uniform labelling in the molecular layer. The intensity of this staining is, however, less than that achieved with anti-NS-4 antiserum when the proportion of staining of molecular to granular layer is compared. Anti-NS-2 and NS-3 antisera also labelled proportionately more of the outlines of granule cells than did anti-NS-4 antiserum. Furthermore, anti-NS-2 antiserum labelled cells

of yet unidentified nature in the white matter tracts.

C. Definition of New Nervous-System Specific Cell Surface Antigens. Several new antisera have been produced: (a) an antiserum to cerebellum of the neurological mouse mutant weaver which detects quantitative differences in antigen expression between normal and mutant cerebella, (b) an antiserum to cerebellar granule cells (in collaboration with Dr. A. Messer), (c) an antiserum to bovine corpus callosum (in collaboration with Dr. G. Campbell, Wistar Institute), and (d) an antiserum to a neural tumor of possibly sub-ventricular cell origin which reacts with fetal and adult brain, kidney and sperm.

D. Immunoselection of Antigen-Positive and -Negative Cell Populations. Pilot experiments have been performed in collaboration with Dr. C. Campbell to investigate the feasibility of using the immunofluorescence activated cell sorter for the automated separation of antigen-positive and -negative cell types. We have been able to improve recovery and viability of cells after the sorting process and have been able to use the antiserum against bovine corpus callosum to isolate cells which are enriched and depleted in glial-cell specific properties (CNPase, S-100 and glial fibrillary acidic proteins).

X. Retrograde Axonal Transport in the Visual System

Our interests in this project center around the incorporation and intraaxonal transport of extracellular material from the region of the axon terminal back to the neuron cell body. This transport mechanism may provide a means by which the neuron communicates with the environment of the axon terminal. We are studying 1) the cytological aspects of the mechanism of retrograde transport, i.e., the organelles that carry the

marker and their relationship with other organelles within the axon and cell body, 2) the characteristics of the phenomenon during development, 3) the effects of different physiological conditions on the transport and 4) the application of the phenomenon as a neuroanatomical tool.

The following results have been obtained in the study of retrograde axonal transport in the visual system. We have confirmed results that transected axons incorporate extracellular marker and transport it sufficiently rapidly to arrive at the cell body before the first signs of chromatolysis appear. During the first hour after transection the axon stump incorporates less extracellular material than does an intact terminal, but by 3 hours cut axons are capable of greater incorporation than that of intact axons. The rate of retrograde axonal transport is not altered immediately after transection.

The following results have been obtained in our study of the use of the retrograde transport method as a neuroanatomical tool. The ^3H -thymidine autoradiographic technique has been used successfully in conjunction with the retrograde transport method to demonstrate the relationship between the time CA 4 neurons are generated in the mouse hippocampus and the axonal connections these neurons eventually make. We have begun to investigate the use of the drug p-chlorophenoxyacetate to remove neuronal lipofuscin and thus avoid the complication of artifactual labeling of neurons in retrograde transport studies.

XI. Cellular Mechanisms of Inherited Retinal Degeneration

The most common inherited disorder causing blindness in man is retinitis pigmentosa, a disease or family of diseases in which the rod photoreceptors of the retina break down slowly and progressively over a period of years. The re-

search in this laboratory has been directed at similar inherited disorders in laboratory animals with the aim of better understanding the basic cellular mechanisms that are affected by various genetic defects. The work is concerned with the interaction of photoreceptor cells and pigment epithelial cells in normal animals and in mutants. Three of our studies are summarized here.

We have discovered that the cerebellar mutant mice, nervous and Purkinje cell degeneration, have a slow, progressive photoreceptor degeneration in addition to the loss of cerebellar Purkinje cells. Although most of the cerebellar Purkinje cells degenerate in the first few weeks of life in these mutants, the retinal lesion evolves slowly over the course of a year. These animals may serve as animal models for some of the many inherited human neurological syndromes in which photoreceptor degeneration is one component.

We have studied the problem of cellular localization of mutant gene action in mice with inherited retinal degeneration,

rd, by using experimental mouse chimeras. Chimeras were produced by fusing eight-cell embryos of albino SJL (*rd/rd*) mice with those of pigmented C57BL/10 (+/+) mice. The eyes of the resulting chimeric mice had patches of normal retina interspersed with patches lacking photoreceptor cells. Pigment epithelial cells of both normal and mutant strains, identified by the presence or absence of melanosomes, were found overlying areas of both normal and degenerated retina. The same findings were obtained using two other strain combinations. The synthesis of rod outer segment discs proceeded normally in photoreceptor cells underlying mutant pigment epithelial cells, as determined by autoradiographic analysis, and phagosomes were found in both mutant and normal pigment epithelial cells. The findings indicate that the pigment epithelial cell is not the primary target of the mutant *rd* gene in the mouse and localize the site of mutant gene action to the neural retina, presumably but not necessarily to the photoreceptor cells. Similar experiments

with *rat* chimeras indicate that the mutant retinal dystrophy gene, *rdy*, acts within the pigment epithelial cell.

Rod outer segment discs are continually renewed at the base of the outer segments, and old discs are shed from the tip of the outer segment and are phagocytized by the pigment epithelium. We have studied outer segment disc "shedding" in relation to the light-dark cycle and have found that a sudden burst of disc "shedding" occurs soon after the onset of light. Within 30 min and for the first 2-3 hrs after the onset of light, many large phagosomes are present in the pigment epithelial cells in albino rats. The number is 3-4 times higher than in the dark immediately before the onset of light. By 4 hrs after the onset of light, throughout the remainder of the 12-hr light cycle and throughout the 12-hr dark cycle, the number of phagosomes in the pigment epithelial cells is low. This phenomenon will be useful for studying the disc shedding mechanism because the process can essentially be synchronized with the onset of light.

Clinical Genetics

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The Relationships Between DNA Replication Kinetics,
Alterations in the Structure of Chromosomes, such as the X, and Human Developmental Anomalies,
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Major Research Projects

I. Genetic Studies of Cultured Cells

One of the premises of modern biology is that all nucleated cells except the gametes contain a full, zygotic complement of genes. The difference in activity of various genes from tissue to tissue and between the different stages of development is the result of gene regulation. An understanding of gene regulation thus becomes essential for an understanding of differentiation and development. In this project, somatic cell cultures are used to mount a number of attacks on the general problem of gene regulation, including a) the control of chromosome replication patterns in hybrid cell, b) the effect of BUDR (5-bromodeoxyuridine) on the expression of differentiated functions and c) the expression of neural tissue antigens in hybrid cells.

A. *The Control of Chromosome Replication Patterns in Hybrid Cells.* Among mammals in general, a cell containing two X chromosomes has only one of them with its

genes in a functional state. The non-functional X chromosome is "inactivated" during fetal life. Coincident with inactivation of the chromosome, the replication of its DNA becomes delayed during the cell cycle relative to that of the functional X. The "turning off" of genes is thus accompanied by, or perhaps the result of, an alteration in DNA replication. A study of the factors controlling DNA replication may therefore provide some insight into the regulation of gene expression. Somatic cell hybrids derived by the fusion of rodent cells with human cells have been selected so that only a very limited number of human chromosomes are present. The replication pattern of the remaining human chromosomes has been found to be indistinguishable from the pattern observed in normal, unhybridized human cells. It is apparent that an intact human chromosome is not required for the control of replication. Moreover, replication of the human chromosomes was not altered by the preponderance of rodent genetic ma-

terial. It may be that control is resident within the individual chromosome, or even chromosome segment. Further insight into this latter possibility will be achieved by a study of hybrids wherein the human genome is provided by different cell types. It has been shown, for example, that termination of DNA replication in several human chromosomes occurs later in fibroblasts than in lymphocytes. If this replication pattern persists in hybrids, then it would seem likely that the replication pattern is "imprinted" in the chromosome.

B. *Effect of BUDR on the Expression of Differentiated Functions.* The DNA synthetic machinery of the cell readily accepts BUDR as a substitute for thymidine, and incorporates this analogue into the DNA. One of the remarkable consequences of the exposure of a cell to BUDR is the general suppression of differentiated functions that results. The manner by which this effect is achieved is unknown, although it has been assumed that somehow the BUDR within the

DNA is responsible. The relation of BUDR incorporation to gene expression has been studied with pigmented hamster cells. In the presence of BUDR, pigmentation of these cells does not occur. When deoxycytidine is also added, pigment synthesis returns. While deoxycytidine lessens the amount of BUDR incorporated into DNA, this cannot be the whole explanation since there is no close correlation between percent of BUDR incorporated and effect upon pigment synthesis. The possibility thus exists that these experiments have uncovered an extra-DNA system upon which BUDR acts in affecting gene expression.

C. Expression of Nerve Tissue Antigens in Hybrid Cells. In the course of differentiation, individual tissues frequently acquire antigenic characteristics that are unique, or nearly so, for the particular tissue. Since these antigens are generally genetically determined, the control of their expression is part of the general subject of gene regulation. Dr. Melitta Schachner (Neuroscience Division, CHMC) has identified a number of antigens which are primarily expressed in nervous tissue. One of these antigens has now been studied in established murine cells of non-nervous tissue and several of them have been found to express the antigen. Hybrids between cells expressing the antigen and other cells not expressing the antigen, have been found to possess the antigen. This type of response differs from results obtained for other neural functions. Because the antigen is expressed in hybrid cells, this system is a most favorable one for analysis of the genetic determinants both of the antigen and its regulation.

II. Visualization of DNA Replication

If the DNA contained in a single human cell were stretched to its full length, it

would measure about one meter. This remarkably lengthy material is coiled, compressed and packaged into the 46 chromosomes. How this vital substance is so precisely packaged, how its integrity is maintained and how it is replicated, are central issues in genetics. One novel approach to these problems which was recently developed in this laboratory is the use of fluorescent dyes which are sensitive to the chemical composition of the DNA. With this technique, it has been possible to explore, a) the details of DNA replication, b) the nature of certain DNA repair processes (sister chromatid exchanges) and c) the occurrence of these events in intact animals.

A. Details of DNA Replication. The demonstration of DNA replication at the level of the individual chromosome has previously been accomplished by the use of autoradiography to reveal the incorporation of tritiated thymidine. Autoradiography possesses the intrinsic limitation that radioactivity can be detected only secondarily, by its action on a photographic emulsion which is some distance removed from the actual location of the tritiated molecule. This produces an uncertainty factor concerning the precise location of the radioactive molecule and reduces the resolving power of the technique. A new method for demonstrating newly synthesized DNA has now been developed which avoids this limitation. This technique depends upon the sensitivity of certain fluorescent dyes, which bind DNA to the presence of heavy atoms such as the bromine of BUDR. Cells which have had their thymidine displaced by growth through several cell divisions in the presence of BUDR are given a short exposure to thymidine during DNA synthesis. The chromosome regions containing the newly incorporated thymidine stain brightly with the fluo-

rescent dye, while the regions containing only BUDR exhibit dull fluorescence. Since the fluorescent light is emitted directly from the dye molecule bound to the DNA, the resolution is considerably greater than that obtained with autoradiography. With this technique, details in the replication patterns of abnormal X chromosomes have been demonstrated that were not detected with autoradiography.

B. Sister Chromatid Exchanges in the "Chromosome Fragility Syndromes." The fluorescent dye technique reveals a second feature in addition to DNA replication. This feature is known as sister chromatid exchanges (S.C.E.) which, as the name suggests, appear to be an exchange of material between two homologous parts of an individual chromosome. When cells are first exposed to some kinds of DNA damaging agents and then examined for the presence of S.C.E., a dramatic increase in S.C.E. frequency is observed. The S.C.E. appears to be the result of repair of the DNA after a particular kind of DNA damage. Enumeration of S.C.E. frequency, both before and after exposure of cells to DNA damaging agents, has become a recognized method for analyzing the hereditary defect in diseases associated with chromosome fragility. In Fanconi's anemia, for example, it has been shown that S.C.E. exchanges occur with abnormally low frequency after exposure to DNA damaging agents. It is possible that the repair system which creates S.C.E. is defective in this disease.

C. Sister Chromatid Exchanges as Evidence of Mutational Events in Tissue Culture Cells and in Spermatogonial Cells of the Mouse. Part of the present day concern regarding environmental pollution relates to the possible mutagenic effects of pollutants. Although a number of techniques are now available to assess the

mutagenicity of various substances, they all suffer one or more disadvantages. The various bacterial systems are rapid and inexpensive, but can only partly take into account the possibility that a given chemical may be converted *in vivo* from a poorly mutagenic to a highly mutagenic substance. *In vivo* testing in the intact

animal, which requires examination of progeny for assessment of mutagenic changes, is incredibly expensive. Alternative approaches which avoid these disadvantages are urgently needed. This need may be at least partly met by the aforementioned dye technique. Procedures have been developed for examina-

tion of S.C.E. in culture cells and, more recently, have been successfully applied to mice. S.C.E. are increased in spermatogonial cells of mice that have been exposed to DNA damaging chemicals, which are known to produce an increase of S.C.E. in cultured cells.

Behavioral Sciences

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Training Academic Teachers and Researchers in the Field of Child Development,
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Dr. Brazelton.

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Major Research Projects

I. Development of Neuropsychological Functions

All neuropsychological studies are cast in

a developmental framework. Results from experimental studies on animals would in most instances not be relevant to the analysis of cortical functions in man, which are of primary interest in

these investigations. Instead, physical maturation, hemispheric specialization, handedness, and sex are used as normal variables, whereas delayed physical growth, specific reading retardation, lan-

guage retardation, endocrine and sex chromosome abnormalities, and localized cerebral lesions in children are used as pathological variables for examining the relation between discrete aspects of perception, cognition and motor action and variations in central nervous system state. Neuropsychological measures are classified on one hand along the *functional* coordinates of serial ordering (temporal order perception, sequential organization of motor actions) and holistic processing; on the other hand along the *structural* coordinates of presumed left and right hemisphere modes of functioning and of interhemispheric cooperation.

In a series of cross-sectional and longitudinal followup studies of kindergarten and school-aged children, we have found that a significant proportion of severely retarded readers, and children at risk for reading failure, show performance profiles which indicate a selective impairment of temporal order perception for auditory and visual information, of serial organization in unimanual and bimanual motor tasks, of memory for sequences, and of other psychological measures presumably associated with left hemisphere strategies of information processing. Many of the same retarded readers perform competently on psychological measures associated with a "right hemisphere mode" of information processing (pattern perception, spatial visualization, holistic processing). In parallel studies, young adult undergraduate and graduate students with persistent dyslexia show similar profiles with impairment in serial order processing and competence in holistic processing; the older as compared to the younger individuals may also have significant difficulties on tasks requiring interhemispheric cooperation.

From these observations we have for-

mulated a set of working hypotheses: (a) The underlying functional domain of impairment in reading retardation is in serial ordering (temporal order perception, memory for serial order, serial organization of sequential motor elements, etc.) and more generally in psychological measures which have been associated with a left hemisphere lateralization; (b) Reading retardation reflects a developmental delay in left hemisphere dependent functions which will be more prominent among late as compared to early maturers (and therefore more prominent in boys than girls); (c) The profile of performance deficits of retarded readers changes in ontogenesis, but the underlying functional impairment persists until adult life. If the findings can be validated in current longitudinal follow-up studies, they will have direct and concrete implications for the large proportion of children of normal intelligence who fail in elementary school.

To refine these hypotheses and to identify the central nervous system mechanism that might be responsible for disturbances in serial ordering behavior, a series of discrete studies have been completed and are in progress:

Dr. Dreier is continuing our earlier studies of non-nutritive sucking behavior in pre-term and full-term normal and high risk infants, and has confirmed the earlier finding that the rhythmic organization of sequential sucking movements is a sensitive measure of impaired central state for serial organization which identifies infants at risk when clinical assessment reveals no abnormalities. He has developed a computer program for relating repetitive motor actions (sucking, respiration, etc.) into Markov processes, and for analyzing the serial organization of motor actions under an information processing model.

Dr. Ferber is analyzing the sequence of behavioral state transitions in young infants, as a refinement on our earlier observation that infants with a history of perinatal distress but no clinical evidence of impairment show marked deviations from the normal in serial patterns of state organization and state transition.

Dr. Waber is testing an earlier finding that patients with Turner's syndrome have a selected impairment of holistic information processing, and are relatively less impaired in the serial organization and analysis of discrete information. At the same time she is analyzing the higher cortical functions of patients with other endocrine and sex chromosome abnormalities, and of normal adolescents, to map out changes in the profile of neuropsychological functions associated with the physiological events of puberty.

Dr. Holmes is examining the profile of neuropsychological performance deficits in children with asymmetries of the two cerebral hemispheres, as demonstrated by computerized axial tomography, and in children with a history of one-sided temporal lobe seizures.

II. Development of Children with Identified Risk Factors

Dr. Walzer's longitudinal study of male infants who were identified at birth as having a sex chromosome variation (XXY, XYY) is in the sixth year of followup. The screening and identification phase of the study has been completed, and the oldest children have passed their fifth birthday. Results to date indicate that 2-year-old children with XXY chromosome complements are not significantly delayed in most developmental milestones when compared to controls. However, XXY boys demonstrate significant delays in expressive language and other aspects of language development, whereas they do not differ from controls on spatial percep-

tion. At 3 years of age, the XXY children continue to demonstrate significant delays in speech and language development as compared to controls; several of the oldest study subjects have developed reading difficulties. These children will be examined in depth at 6-7 years of age for the neuropsychological functions outlined in previous paragraphs, to specify the nature of the functional deficits associated with language impairment in terms of cerebral functions. Dr. Walzer's data indicate no significant correlations between social class and the incidence of abnormal sex chromosome karyotype (XXY, XYY), but suggest that the incidence of severe chronic upper respiratory disease may be significantly higher in such children than in the general population.

III. Developmental Effects of Low Lead Level Exposure

This program project is a coordinated clinical, epidemiological and experimental investigation of the effects of low doses of lead upon the physiological and psychological development of humans and laboratory animals.

Human Studies: The major study is a large scale retrospective cohort study of 2,000 school-aged children considered asymptomatic with respect to lead toxicity. The lead burden in these children is measured by analyzing lead in shed deciduous teeth, a method shown by us to reflect earlier exposure when more conventional measures, such as blood lead levels, may be within normal limits. With the cooperation of school systems and health departments adjacent to Boston, we are collecting teeth from first-graders. The teeth are then embedded and sliced, and specific zones of dentine extracted and analyzed for lead. Children with the highest levels, and controls with low lead burdens, are identified and brought to

the neuropsychological laboratory for testing using a battery of instruments measuring intelligence, verbal performance, perception, and motor function. A subgroup will also have a speech and hearing battery and quantitative electroencephalogram. Other variables known to affect development, such as birth weight, socioeconomic status, sex, and race, are stratified in the analysis, and their contribution to outcome measured.

Experimental Studies: The purpose of these studies is to produce animal models in which lead dose is systematically manipulated as the independent variable, and to examine the neuroanatomical, physiologic and neurochemical changes that accompany lesser doses of lead.

Progress has been made by Dr. Lorenzo's group in developing a low dose model in the newborn rabbit. Rabbits aged 1 to 30 days have been exposed to lead in the following concentrations: 1, 4.5, 9, and 18 mg/day. At all doses, lead fed animals could be distinguished from controls. No weight changes were observed at 1 mg/day, nor were histologic changes seen in the brain. At all doses, basophilic stippling was seen in the peripheral smear. Animals receiving 4.5 mg/day or more demonstrated histologic changes in the brain; the severity was dose related.

Immature rabbits were given lead-203, a gamma emitter with a half life of 52 hours. Studies of accumulation of the isotope demonstrate that administration prior to the fifteenth day of life yields higher counts in the caudate and cerebellum than later administration. This finding is in agreement with other studies that suggest that younger organisms treat lead differently and are more vulnerable to its effects.

Dr. Averill has been studying the effects of low doses of lead on cortical histogenesis in the rat, employing light microscopy and quantitative measures of ultrastructural change. After preliminary studies using light microscopy on brain tissue of rat pups exposed to lead via mother's milk were accomplished, the highest dose which produced no histologic change was defined as the low level dose. This was 1% lead carbonate in the mother's chow. Offspring of mothers fed 1% PbCO₃ and pair-fed controls were examined at 10 days of life. The mean blood leads for experimental animals was $93 \pm 15 \mu\text{g}/\text{g}$, controls $9.0 \pm \mu\text{g}/\text{g}$. No significant changes were found in body or brain weight; brain histology; swimming or eye-opening time; dendritic numbers in Layer V, frontal cortex; dendritic branching index; EPTA stained synapses, Layer I frontal cortex; synaptic maturity profile; or ultrastructure of neonatal cortical capillaries. The mean number of total synaptic spines per neuron were 8.52 ± 1.34 for lead fed animals, and 3.97 ± 0.75 for pair-fed controls. Two more cohorts of rats raised on the same dosage level, but sacrificed at 21 and 40 days, will be studied to determine the effects of lead later in the course of histogenesis.

IV. Studies on Mother/Infant Social Interactions

Under Dr. Brazelton's direction, a group of investigators applying the Brazelton neonatal assessment scale to normal and small-for-dates infants, have found that small-for-dates infants differ significantly from normals, particularly on behavioral items contributing to social interaction, and that a number of these infants develop psychosomatic disturbances towards the end of the first year of life. The investigators are also analyzing in detail the social interaction of young infants and their caretakers. A coding sys-

tem for interactional analysis has been developed and applied to patterns of mother/infant interaction when specific features of the interaction are experimentally altered. Findings to date document the importance not only of the signals

given by the mother to the child, but also those given by the child in regulating the social interactions of the parent/infant couple.

Dr. Demos has recently begun an independent study of affect expressions as

mechanisms that regulate the mother/infant transactions in 6-, 12- and 18-month-old infants, using a method of interaction analysis based on an ethological model and the categories devised by Sylvan Tomkins.

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Financial Support:

Histochemistry of Phosphodiesterase in the Retina, NEI, \$53,545, Dr. Robb.

Major Research Projects

I. Cyclic Nucleotide Phosphodiesterase in the Retina

The primary project at present is a histochemical study of cyclic nucleotide phosphodiesterase in the retina. This enzyme has been identified biochemically in photoreceptor outer segments and may be light sensitive under certain conditions. This histochemical approach to its localization has been helpful in identifying enzyme activity on rod outer segment lamellae. Current studies are aimed at identifying the enzyme in a strain of mice with hereditary retinal degeneration and in investigating possible differences in enzyme distribution in human (autopsy material) rods and cones. A lead precipitation technique is employed with cyclic AMP and cyclic GMP as substrates.

Precipitation patterns are studied with the electron microscope.

Other exploratory projects of shorter duration include a study of histopathologic changes in the lenses of patients with Down's syndrome, a morphological study of the effects of the ocular retardation (or) gene in an inbred strain of mice, and a light and electron microscopic study of myelin formation in the optic nerves of infants and children of various gestational and post-natal ages. A final clinical-pathological study concerns the correlation of pathological changes in the eyes of children having acute leukemia with the state of their systemic illness at the time of death.

II. Electrophysiology of Eye Movements

The electro-oculogram measures changes in the standing potential of the eye with respect to skin electrodes during eye

movements in appropriate directions. Eye movement velocity and rates of acceleration can be determined from such data. Present studies are directed toward measurement of oblique eye movements corresponding to the direction of maximal action of the inferior and superior oblique eye muscles. Over- or underaction of these muscles are commonly held to account for vertical incomitance in strabismus, yet there is at present no information by which to judge whether the underacting muscle is truly paretic (slow acceleration and reduced velocity). Similar studies are being undertaken on patients with congenital esotropia to determine whether sixth cranial nerve palsies underlie the commonly noted abduction deficiency.

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Financial Support:

Protein Structure and Collagen Maturation, NIAID, \$38,418, Dr. Gallop.

Fibroblasts and Connective Tissues in Development, NIA, \$124,906, Dr. Gallop.

The Biology of Skeletal and Other Connective Tissues, NIAMDD, \$554,820, Dr. Glimcher.

Separation and Characterization of Lung Elastin, NHLI, \$175,077, Dr. Gallop.

Elastin Synthesis by Cultured Lung Cells, NHLI, \$29,986, Dr. Hauschka.

Ultrastructural Analysis of Bone Cell Metabolism, NIAMDD, \$40,770, Dr. Holtrop.

Molecular Basis of Joint Lubrication, NIAMDD, \$86,994, Dr. Radin.

Mobil Oil Foundation, \$2,500, Dr. Radin.

New England Peabody Home For Crippled Children, \$50,000, Dr. Glimcher.

Hemoglobin A1C in Patients with Diabetes Mellitus, NIAMDD, \$8,190, Dr. Gallop.

United Order True Sisters of Noemi, \$10,000, Dr. Glimcher.

Liberty Mutual Insurance, \$21,200, Dr. Glimcher.

Overall Objectives and Outline of Research Projects

In the most general terms, the broad, long range objective of this program is to obtain a better understanding of the fundamental structure, organization and biological functions of connective tissues by a multidisciplinary approach, with particular emphasis on those tissues comprising the skeletal system. While a

major portion of the research effort will be devoted to particular tissues such as bone and cartilage, considerable effort will also be made in the study of connective tissues in general. It is hoped that by diversified but concentrated research effort on the fundamental aspects of connective tissues at the molecular, tissue, organ and animal levels, we will be able to approach both diagnostic and therapeutic problems related to clinical pathol-

ogy with a greater understanding of the underlying basic processes leading to disease.

Major Research Projects

I. Studies Related to the Mechanism of Calcification

A multidisciplinary research effort is being directed at elucidating the molecular structure of the initially deposited mineral phase in bone and other calcified

tissues and the changes which occur in the bone and mineral during aging and maturation. Bone of various ages and maturation is prepared by a density centrifugation method and a mass distribution of bone of various densities plotted for animals of different ages. Each of these fractions is then subjected to wet chemical analysis, a variety of x-ray and electron diffraction techniques, electron microscopy, electron probe microanalysis and electron spin microanalysis. In addition, aliquots of the same samples are being studied by a variety of biochemical techniques to follow parallel changes which are occurring in certain biochemical parameters of collagen and the proteoglycans.

In addition, new techniques have been developed and are now being expanded in which bone and other mineralized tissues are being examined in the electron microscope and by electron diffraction and electron probe microanalysis without being subjected to aqueous solvents. This is in order to avoid phase changes induced in the mineral phase by aqueous solvents. Two of the methods, namely the use of organic solvents and the use of freezing techniques without any application of aqueous or nonaqueous solvents at all, have already been explored in some detail. Definite changes have been observed in the electron microscopic appearance and in the structural characteristics of the bone mineral using these methods of preparation as opposed to the classical methods utilizing aqueous solvents and stains. Further work is being pursued along these lines using a variety of nonaqueous solvents. It should now prove possible to explore small but important changes in the mineral phase as a function of aging and maturation, as noted above.

Within the past year a new amino

acid, γ -carboxyglutamic acid (Gla) has been identified in a small protein present in bone matrix. This peptide differs from prothrombin and the other blood proteins which had previously been shown to contain this calcium binding amino acid in a number of respects. Efforts are now being made to isolate sufficient amounts of this peptide in order to do a complete amino acid sequence. The calcium binding peptide containing Gla has also been shown to contain *o*-phosphoserine and *o*-phosphothreonine which would assist in its calcium binding. Studies are also in progress delineating its interaction properties with calcium both by physical chemical methodology and by direct measurement using electrophoretic and electron spin resonance techniques.

A number of tissues such as calcified atherosclerotic plaques, kidney stones, and so forth are also in the process of being investigated for the possibility that they contain either the same peptide as that which occurs in bone or another peptide containing Gla. To date the calcium binding amino acid Gla has been identified in the EDTA extracts of atherosclerotic plaques, calcified heart valves, calcified skin, and certain kidney stones.

II. Studies Relating to the Structure, Development and Maturation of Collagen and Studies of Certain Connective Tissue Disorders

A major effort is being made in the laboratories to characterize the collagen from a number of the connective tissues particularly bone and cartilage. These include the mapping out of the distribution of various molecular species, i.e., the proportions of types I, II, and III in the various tissues, and any changes in this distribution which may occur in skeletal and connective tissue diseases.

In addition, various of the post-translational modifications which occur in the genetically different collagens are being examined in great detail. These studies include the isolation and identification of both reducible and nonreducible crosslinks and their structural characterization by mass spectroscopy. Other studies are concerned with the pathways and methods of biosynthesis and maturation of these crosslinks utilizing cell culture, organ culture, tissue culture and *in vivo* methodologies.

The connective tissue from patients having a wide variety of skeletal and connective tissue diseases is also being systematically examined with regard to changes both in molecular species and post-translational modification. These diseases include osteogenesis imperfecta, Dupuytren's contracture, idiopathic scoliosis and so forth. To date definite changes in the maturation of crosslinks have been noted in all of the connective tissues in patients with osteogenesis imperfecta, and a number of other changes noted in the palmar fascia of patients afflicted with Dupuytren's contracture. The latter changes include an increased hydroxylation of certain of the lysine residues of collagen and an increase in the ratio of type III to type I collagen in the palmar fascia.

III. Studies Relating to the Formation, Resorption and Repair of Bone and their Relationship to Certain Diseases of Bone

A group of studies is currently being carried out using the parabiotic method of Ross *et al.* in order to determine the progenitor origin of bone forming and bone resorbing cells. After induction of new bone formation or the resorption of new bone in one of the parabiotic animals, H^3 -thymidine is injected to trace the origin of bone forming or bone resorbing cells. Parathyroid hormone is

also used to increase stimulation of bone resorption. Various blood and tissue samples (spleen, liver, intestine, marrow) are then examined by both light and EM radioautography and scintillation counting.

In addition to these studies, other morphological studies are being pursued by quantitative electron microscopy on the changes occurring in osteoclasts when bone resorption is stimulated. These have shown a definite increase in ruffled borders as opposed to clear zones when bone resorption is activated. Parallel chemical studies of bone resorption are also being carried out and the isolation of a bone collagenase has been accomplished. Studies are now in progress characterizing the properties of the bone collagenase and its method of action in various biological and chemical regulatory mechanisms which control its production, synthesis and activity. In addition, attempts are being made to produce antibodies to the enzyme in an effort to localize the cells which synthesize the enzyme.

IV. Studies on the Wear and Mechanical Properties of Articular Cartilage, with Particular Emphasis on Degenerative Changes in Cartilage such as Osteoarthritis.

Paralleling the biochemical studies of the molecular changes in collagen of cartilage with aging, maturation and degeneration, mechanical studies are also under way delineating the material properties of cartilage as a fabric. These include the

characterization of the viscoelastic and wear properties of cartilage *in vitro* as well as the changes which occur in the collagen and proteoglycans during experimentally induced osteoarthritis produced by continuous and intermittent stresses across the joint.

The *in vitro* studies on the mechanical and wear properties of cartilage as a tissue fabric are also being correlated with changes in the chemical properties induced by crosslinking of the macromolecular components as well as by selective extraction and/or degradation of certain of the component structures. All of these studies are being combined with theoretical and mathematical approaches in an effort to describe the system in more quantitative terms. These latter studies are being carried out in collaboration with the Department of Engineering and Theoretical Mechanics at Rensselaer Polytechnic Institute.

V. Studies on Elastin from Normal and Pathological Lung Tissues

Elastin is prepared by new and mild methods developed here from anatomically defined portions of calf lung. This elastin is characterized and the maturation of crosslinking profiles determined. Human lung from normal and pathological states are investigated when available from fresh autopsy material. A new fluorescent histological technique for studying elastin crosslinking intermediates has been developed and is being applied to the examination of pathological tissues.

VI. Studies on Fibroblasts and Connective Tissue in Developmental Aging

The aging of diploid human fibroblasts in culture is being studied. Collagen production and modification by various cell strains as a function of passage level is examined using various cofactors and inhibitors. These processes are cell age dependent: "young" cells (early passage level) require ascorbic acid for complete collagen prolyl hydroxylation, whereas "old" cells (late passage level) appear to lose this dependence and carry out only incomplete collagen hydroxylation with or without ascorbate. Drugs such as hydralazine and diphenylhydantoin have interesting effects on this system. Prolyl and lysyl hydroxylases are being isolated from cells at different passage levels and are under investigation. Among the cells employed are fetal strains such as WI-38 and more recently IMR-90, as well as cells derived from explants of normal and pathological tissues.

In order to define a meaningful measure of cell "age", experiments are underway in which certain aspects of the purine metabolism of the cells are examined, in particular the salvage pathways and *de novo* purine synthesis. The working hypothesis is that enzyme coded for on the X-chromosome may show a more rapid age dependent decay than enzymes coded for on autosomes. Other X-linked systems will also be examined in the "aging" diploid human fibroblast.

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Financial Support:

The Effects of Irritation and Drugs on Airway Epithelium – An Experimental Study of Mechanisms,
Council for Tobacco Research, \$100,000, Dr. Reid.

Combined Neuropathologic and Epidemiologic Study, NINDS, \$618,435, Dr. Gilles.

Reactions of Perinatal and Developing Brain to Insult:
Endotoxin Model of Perinatal Telencephalic Leucoencephalopathy,
United Cerebral Palsy Foundation, \$36,642, Dr. Gilles.

Major Research Projects

I. The Effects of Irritation and Drugs on Airway Epithelium – an Experimental Study of Mechanisms

The airway epithelium, part of the surface of the body, is affected directly by irritants and pollutants. Two aspects of this response are of major importance to human disease – (i) hypersecretion of mucus accompanied by increase in the number of mucus secreting cells in the surface epithelium and the size of the submucosal gland and (ii) disturbance of cell division. These can be related respectively to chronic bronchitis and bronchial carcinoma, two conditions which have been linked to the habit of tobacco smoking. The increase in goblet cell number and submucosal gland size are seen also in childhood disease. Mucus hypersecretion, increased mitotic activity and various other features of airway epithelium response we now know are associated with human disease and can be produced

experimentally in the rat.

These are the effects of 'irritation' but some of them can also be produced by neuromimetic pharmacological agents which suggests the mechanism by which irritation may produce its effect. It is of additional value for the purpose of our experiments that in some aspects the drugs do not exactly mimic irritation.

We have developed quantitative methods for identifying the various changes in the structure and function of airway epithelium so that the severity of any change can be assessed and the inhibitory effect of a given agent detected and measured.

Using the results of these previous studies we are now able to turn to the experimental analysis of the various mechanisms involved in the response to irritation and to identify features that are specific to a particular irritant and those that are more general.

The changes caused by inhalation of tobacco smoke will be compared with

those caused by various pharmacological agents, including nervous system mediators, to identify those features of the response to irritation that could be mediated by a particular nervous pathway.

It will be established whether the changes, produced by irritation, in the airway epithelium are prevented or diminished by antagonists and blockers of the neuromimetic drugs thereby indicating the mechanism of their induction.

The effects of anti-inflammatory agents will be compared with that of drug antagonists. In so far that their effects are similar it will suggest the way the anti-inflammatory agent may be working; in that they are dissimilar, the presence of an additional mechanism is identified.

II. Studies on Myelin Development

For several years we have been investigating the location and incorporation of long-term stable components of the nervous system using autoradiography. With Drs. Uzman, Rawlins and Salpeter we were able to demonstrate that ^3H -

labeled cholesterol incorporated into peripheral nerve was not limited to myelin sheaths as had been suggested. Recently by adapting a method of embedding tissues for electron microscopic examination without exposure to organic solvents, it was shown that ^3H -cholesterol incorporated into the central nervous system during development is also not limited to myelin sheaths in the adult. It was noticed during these studies that ^3H -cholesterol is apparently concentrated in cerebral endothelial cells and we are now further investigating this phenomenon and comparing it with ^3H -cholesterol transit across vessel walls in other organs. In association with these studies we have been attempting to develop methods for increasing the efficiency of the autoradiograms such as scintillation and double-layers of emulsion but without much success. We (Drs. Martinez, Willis, Davis, Treves and Judy of Nuclear Medicine) have applied frozen section autoradiography for $^{99\text{m}}\text{Tc}$ and developed methods for calculating microdosimetry of these compounds.

With Dr. Daniel Kirschner of Brandeis University, we have demonstrated that the crystalline structure of myelin varies with age, as does the biochemical composition.

Dr. Roderick T. Bronson has been comparing the effects of enucleation and exenteration of the orbit on the development of rat cranial nerves. He has found some consistent differences in fiber size distribution between right and left rat cranial nerves such that the potentially small effects of enucleation on growing and myelinating cranial nerves are no greater than the innate variations. He has been able to develop methods for collecting and analyzing data on nerve fiber size distributions to counteract the major problems inherent in analyzing data with

a bimodal distribution.

III. Blood Brain Barrier and Seizures

In collaboration with Dr. Lorenzo, we have been seeking the site of the protein leak which occurs in the brains of cats with experimentally induced seizures. We have demonstrated that the protein (horseradish peroxidase in our experiments) localizes in the extracellular space of the brain and accumulates in a manner which suggests diffusion after the initial leak. The leak occurs across vessels larger than capillaries, probably arterioles. These studies may be important to an understanding of the transient neurological signs which follow severe seizures in man, e.g. altered consciousness.

IV. Endotoxin encephalopathy in the Neonate

In the human neonate we have demonstrated a relationship between terminal bacteremia and a white matter abnormality, perinatal telencephalic leukoencephalopathy. This led to the speculation that immature white matter was vulnerable to endotoxemia. This speculation has been confirmed in the neonatal kitten. Current experiments have demonstrated that immature telencephalic white matter of the neonatal kitten undergoes necrosis after a single exposure to the 055:B5 strain of *Esch. coli* at 50 $\mu\text{g}/\text{mL}$, given intraperitoneally. Furthermore, this necrosis appears unrelated to cardiovascular collapse or to a transient disseminated intravascular coagulopathy. Long term follow-up of the large cystic lesions present two weeks following the exposure to endotoxin indicates that the cysts disappear leaving only white matter scars.

V. Evaluation of Childhood Cerebellar Tumor Biopsies

Observer variability in the recognition of each of 16 histologic features ranged from

0.68 to 1.00 as estimated by the Index of Adjusted Agreement. The relationship of histologic features, as seen in the first biopsy, to survival was studied by the use of life tables. A simple classification system which accentuates differences in survival based on the clustering of histologic features was then developed. Two major subgroups of cerebellar gliomas were identified. They have different patterns of clinical features, markedly different 10 year survivals, and differing clusters of histologic features. A multivariate analysis based on 42 histologic and clinical features further diminished the classification error to 4% as to life or death 10 years following initial operation. Studies of temporal changes in the histologic composition of cerebellar gliomas at Children's Hospital Medical Center between 1927 and 1968 indicates a marked change in histologic patterns with time. One histologic feature (oligodendroglial foci) clustered within a short span and had a survival rate (100%) and male sex proportion which was quite different from the other histologic features.

VI. Collaborative Perinatal Project of the NINCD

A series of anatomic and epidemiologic studies is being completed. The epidemiologic studies are, for the most part, case-control studies which explore the associations between common events within the developing brain such as hemorrhage and necrosis and potential antecedents or constellations of antecedents. Other studies derived from this material have explored growth functions and developed a growth model for the human fetal brain, have identified the end of the second trimester as a period of great importance to the developing human fetal brain, and have developed an alternative measure of developmental time based on measured associations between myelin-

ating tracts which does not rely on the vagaries of estimated gestational age for its abscissa. Patho-anatomic studies including the development of gyral patterns, surface area, germinal mass, dorsal mesodiencephalic junction, telencephalon medium, hemorrhage, necrosis, dysraphism, and malformation have been completed.

VII. Altered Structures and Function of the Renal Glomerulus in Autologous Immune Complex (AIC) Nephritis

Over the past four years, the electron microscopy laboratory in the Department of Pathology has been largely involved in the study of the ultra-structural basis for the altered permeability of the renal glomerulus in experimental autologous immune complex (AIC) nephritis. In addition human biopsy material is examined for diagnostic purposes as well as to determine whether there are alterations in the structure of the slit pore diaphragm (see below) in the nil disease variant of the nephrotic syndrome of childhood. And finally, since the acquisition of a Balzer's freeze etch unit, the structure and development of intercellular junctions in the alveolar-capillary membrane is being examined.

The initial studies on the increased permeability of the glomerulus in AIC nephritis were based on the assumption that there are two permeability barriers to protein in the glomerular capillary wall: a) the glomerular basement membrane (GBM) which acts as a coarse filter and b) the slit pore diaphragm (SPD) which acts as a fine filter. Using enzymatic tracers of varying molecular weight as well as ferritin, it appeared that there was an increase in permeability of the GBM to ferritin in those areas in which immune complex deposits were present. The intervening, uninvolved GBM showed no detectable alteration in permeability.

However, whether an additional defect existed in the SPD could not be resolved using conventional fixation techniques for electron microscopy. In order to visualize the isoporous substructure of the SPD, kidneys were fixed by perfusion with tannic acid-glutaraldehyde. Under these conditions it was found that in the early stages of the disease, when proteinuria is relatively mild and the immune complexes small, there is no intrinsic defect in the SPD. Later, when proteinuria is massive, the immune deposits large, and there is extensive foot process spreading, there is a reduction in podocyte surface area with a consequent folding and buckling of the redundant SPD. These changes in the morphology of the SPD are regarded as secondary to proteinuria and not the underlying cause of it. Instead, a few slit pore complexes lacking an SPD altogether, or areas of foot process detachment appear to be the sites where massive protein leakage can occur.

Since the initial tracer studies in AIC nephritis it has become clear that the interpretation of such studies in kidneys fixed by immersion may be misleading largely due to diffusion of the tracer which may occur before adequate fixation takes place. This problem has now been overcome by using Munich-Wistar strain rats which have superficial glomeruli immediately below the renal capsule. These glomeruli can be rapidly fixed *in situ* in the living anesthetized animal. Furthermore, to eliminate the injection of foreign proteins as tracers into the circulation, the location within the glomerulus of the animal's endogenous circulating serum proteins is identified by immunocytochemical means. This is done by using rabbit anti-rat Fab fragments of anti-albumin tagged with horseradish peroxidase. The location of the endogenous albumin is then identified

cytochemically. In this way the abnormal distribution of endogenous serum proteins can be studied. Such experiments in Munich-Wistar rats having AIC nephritis are in progress.

Although the highly ordered substructure of the SPD was first studied in murine kidneys, we have established that a similar structure is present in the human kidney. While its function is still a matter of controversy, it is regarded by many as a structure which controls hydraulic flux across the glomerular capillary wall. Any primary defect in its structure could change the hydraulic flux across the GBM in such a way that protein molecules could be swept into the urinary space by bulk flow. Whether such a primary defect in the SPD exists in the nil disease variant of the nephrotic syndrome remains to be established. At present selected renal biopsies from patients with this disease are currently being investigated.

A further approach to investigate the function in protein filtration of the various anatomical barriers (GBM and SPD) in the glomerulus is to correlate the time of their first appearance with the initiation of glomerular filtration. The latter will be ascertained in kidneys of newborn Munich-Wistar rats fixed *in vivo*. The location of circulating endogenous serum albumin will be identified by using the immunocytochemical methods described above. A careful correlation of the time of formation of the GBM and the first appearance of the SPD with the location of endogenous serum proteins will be of value in understanding the part that these anatomical structures play in glomerular filtration of protein.

The role of the mesangium in glomerular function in general, and in AIC nephritis in particular is still unclear. Preliminary studies in this laboratory show

that, in contrast to two other proteinuric models (aminonucleoside nephrosis and nephrotoxic serum nephritis), there appears to be a depression of mesangial phagocytic function in AIC nephritis. This altered mesangial function is being studied at present with intravenously injected colloidal carbon and the distribution of carbon is examined over varying periods of time by light microscopy and electron microscopy. Parallel quantitative measurements of serum and tissue carbon levels are also being carried out.

VIII. Ultrastructure of Intercellular Junctions in the Alveolar-Capillary Membrane

Based on data obtained from numerous physiological experiments, as well as ultrastructural tracer studies, it is clear that it is the epithelial rather than the endothelial cell layer which constitutes the chief barrier to diffusion of small water soluble molecules across the alveolar-capillary membrane. Studies carried out in this laboratory have shown that by freeze fracture the pulmonary epithelial junctions are composed of a continuous network of 3-5 interconnected strands on the PF face and complementary grooves on the EF face. This is in contrast to the capillary endothelial junctions which are composed of 1-3 partially interconnected rows of particles which show occasional interruptions.

The junctions in the venular portions of the capillary bed are even more tenuous. Currently studies are in progress using fetal lamb lungs in which radioisotope measurements have been carried out to determine the permeability of the alveolar capillary membrane at various stages of fetal development. The lungs are then fixed and subjected to the freeze fracture technique in order to correlate junctional structure with the functional measurements.

IX. Effect of Lead on Rat Cerebral Cortex

We have determined a maternal dietary lead concentration which results in slowed neonatal growth and are analyzing the effect of this slowed growth on neocortical neuron dendritic complexity, and synaptic spine formation. Total E-PTA stained synapses, synaptic maturational indices and capillary morphology are also compared to pair-fed, ad-lib fed and ad-lib fed lead-dosed animals at 10, 21, 40 and 60 days of age. Behavioral measures (made at various time intervals) include eye opening onset, startle reflex onset, latency to coordinated swimming activity, Thorndikian T-maze tasks and open field activity.

X. Ventral Horn Cell Synaptology in the Normal C57B16 Mouse and the Wobbler Mouse

We are presently characterizing and tabulating the anatomic features of somal

axon terminals of cervical spinal cord ventral horn cells by quantitative electron microscopy. The purpose of this project is to determine the temporal sequence of axon terminal loss with respect to ventral horn cell degeneration in the inherited wr/wr trait. Preliminary data suggests that boutons are either not formed and/or degenerate prior to the somal degeneration.

XI. Effects of Neonatal Endotoxin upon Developing Mammalian Telencephalic White Matter

Endotoxemia results in white matter alterations homologous to those occurring in so-called perinatal telencephalic leukoencephalopathy of children. Current work includes the effects of various pharmacologic agonists and antagonists for this drug-effect to determine suitable treatments for this lesion.

XII. Neuroparmacology and Ultrastructural Evolution of Inherited Striocerebellar Degeneration in the Kerry Blue Terrier

The clinical effects of various neurotransmitters and their antagonists upon this progressive Huntington's disease-like model are being studied with regards to regional tissue concentrations of the neurotransmitters and their enzymes and the light and electron microscopic appearance lesions which evolve in the caudat nucleus, substantia nigra, inferior olivary nucleus and cerebellum.

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Financial Support:

A Bromine-77 → Selenium-77m Generator, NHLI, \$44,400, Dr. Treves.

A 191Ios - 191mIr Generator For Radionuclide Angiocardiology, NHLI, \$4,000, Dr. Treves.

Amniopague Study, Sterling-Winthrop, \$6,000, Dr. Kirkpatrick.

Radionuclide Assessment of Regional Lung Function in Babies and Small Children,
James Picker Foundation, \$7,500, Dr. Treves.

New England Nuclear Corp., \$20,000, Dr. Davis.

Major Research Projects

I. Radionuclide Assessment of Regional Lung Function in Children

This research is directed toward the development of a radionuclide method for evaluation of regional lung function in small children using radioactive inert gases. Designs and construction of special magnifying collimators for the smallest children are included within the objectives of this research. We have recently developed a suitable method for regional lung function determination on children and have completed the evaluation of commercial and experimental magnifying collimators.

We are studying selected groups of patients with congenital heart defects (pre- and postoperatively), cystic fibrosis, diaphragmatic hernia, pectus excavatum, etc.

II. A Bromine-77 → Selenium-77m Generator of Angiography

The objective of this research is similar to that described in the Osmium-191 Iridium-191m project. Bromine-77 has a physical half-life of 56 hours and Selenium-77m has a half-life of 18.1 seconds and decays with gamma emission of 162 KeV. Bromine-77 is produced in a cyclotron. A common characteristic to both generator systems (Os-Ir; Br-Se) are that they result in improved diagnostic information and reduction in the radiation

dose. In addition, they can provide a continuous source of ultrashort-lived radionuclides in medical centers remote from the production site.

The research with Bromine-77 → Selenium-77m generator is in its embryonic stages. With the arrival of a nuclear chemist this summer, it is expected that considerable progress will be made toward the development of the generator.

III. Development of an Ultrashort-Lived Radionuclide Generator for Angiocardiology

This research is directed towards the development of ultrashort-lived generators, particularly Osmium-191 → Iridium-191m, for radionuclide angiocardiology.

raphy in children. Osmium-191 is produced in the reactor and has a physical half-life of 15 days. Osmium-191 decays to iridium-191m with a half-life of 4.9 seconds and a gamma emission of 129 KeV. The osmium-191 is absorbed on an anion exchange resin which does not bind iridium-191m. Iridium-191m can be eluted repeatedly (every 2 minutes) from

the generator and used for angiography.

The availability of this radionuclide will result in increased information plus a significant reduction of the radiation exposure to patients. The radiation dose can be reduced by a factor of 500 to 1000 compared to presently available radionuclides used for the same purpose.

This research is progressing well. We

have built a small number of prototype generators and carried out experimental angiocardiology on rhesus monkeys. Currently, we are in the process of studying the body distribution and toxicity of the generator products with the ultimate objective to develop a system suitable for human use.

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Financial Support:

Monsanto Corporation, \$227,218, Dr. Folkman.

Alza Corporation, \$10,000, Dr. Folkman.

Antiangiogenesis—New Method of Therapy of Solid Tumors, NCI
\$104,069, Dr. Folkman.

Major Research Projects

I. Studies on Tumor Angiogenesis

Cancer biology is the major interest of this laboratory. We are primarily concerned about those properties which a transformed cell must acquire before it can become tumorigenic. Of these properties, the most important seems to be the capacity of a tiny tumor nodule to induce new blood vessels to grow from the host. Prior to vascularization solid tumors remain in a viable but dormant phase at diameters of 1 to 2 mm. We have shown that solid tumors elaborate a soluble extract, tumor angiogenesis factor (TAF), which elicits capillary proliferation in the host.

Current investigations include: 1) Purification of TAF, 2) Purification and characterization of a factor from cartilage which inhibits capillary proliferation, 3) Development of slow release polymers for assay of both TAF and cartilage derived inhibitor, 4) Endothelial cell and capillary growth in culture for use as an *in vitro* assay, 5) Effects of geometry on

cell growth, 6) Response of host endothelial cells to tumor and TAF, 7) Regression of capillaries, 8) Studies of a cartilage derived growth factor and stimulation of DNA synthesis in chondrocytes, 9) In addition, there is a long term investigation, separate from the tumor angiogenesis problem, concerned with the role of platelets in neutralization and clearance of endotoxin.

A. Purification of TAF. This project is being conducted in collaboration with Dr. Bert Vallee's group of the Peter Bent Brigham Hospital. Tumor cells are grown in spinner flasks to a density of 2.5×10^6 ml. The medium is withdrawn, salts removed by dialysis, and the resulting protein solution is lyophilized. Gel exclusion chromatography, ion exchange chromatography, and solvent partitioning are utilized; each yielding a different discrimination among the species present. Successive application of these techniques has recently been possible because of increased production of the starting material from scale-up of tissue culture. Assays of available fractions are carried out

on the chorioallantoic membrane of the chick embryo and in the rabbit cornea.

B. Purification and Characterization of a Factor from Cartilage which Inhibits Capillary Proliferation. Crude cartilage extracts have been shown to inhibit tumor angiogenesis in the rabbit cornea. Cartilage from approximately 200 pounds of calf scapulae extracted with guanidine hydrochloride, dialyzed, lyophilized, and passed over a chromatography column produces a few milligrams of active fraction. This fraction will be further purified using gel electrophoresis and isoelectric focusing. All fractions are being tested for ability to inhibit tumor-induced angiogenesis in the rabbit cornea assay. Upon accumulation of sufficient purified inhibitor, a therapeutic trial in rabbits using prolonged infusion will be undertaken.

C. Development of a Slow Release Polymer for Assay of Both TAF and Cartilage Derived Inhibitor. A variety of polymers have been tested for their ability to release biologically and biochemically active macromolecules for prolonged periods. Three of these have been found to be com-

pletely non-inflammatory in the rabbit cornea and also release microgram quantities of protein from implanted 1-2 mm pellets for periods up to 100 days. Elvax 40 (ethylene-vinyl acetate copolymer) is being used to release TAF in the cornea, so that strength dilution curves can be made for purified fractions. Elvanol (polyvinylalcohol) is being used similarly on the chorioallantoic membrane. Current studies are focused on the characteristics and mechanism of release of macromolecules from these polymers.

D. Studies of Endothelial Cell and Capillary Growth in Culture for Use as an In Vitro Assay. Studies over the past three years have demonstrated that neither serum nor TAF will stimulate growth or mitosis of human umbilical vein endothelial cells at confluence. However, recent studies indicate sparsely cultured endothelial cells may survive and proliferate in the presence of medium enriched with TAF but not without it. Efforts to develop an assay for TAF on this basis are being undertaken. The ability to successfully culture capillaries would provide a powerful tool for the assay of both TAF and inhibitor, however, this has thus far eluded this and all other laboratories. Studies of capillary proliferation from the eggs of certain chick embryo tissues, growth of brain capillaries, and the role of collagen in promoting capillary growth are being pursued.

E. Studies of the Effects of Geometry on Cell Growth. A long term study is underway to determine the effects of geometry on the control of cell growth at the level of individual cells and at the level of cell populations. This study is being conducted in collaboration with Professor Harvey Greenspan of MIT, using time-lapse photomicrography and mathematical models.

F. Studies of the Response of Host Endothelial Cells to Tumor and TAF. Preliminary data from studies in the rabbit cornea suggest that in addition to mitosis, host endothelial cells respond to tumor implants and TAF by migration out of existing blood vessels toward the implants. Mechanisms of endothelial cell response are being studied with autoradiography, scanning and transmission electron microscopy, and slit-lamp stereomicroscopy.

G. Studies of the Regression of Capillaries. New capillaries which have formed in response to tumor or TAF regress and disappear when the TAF stimulus is removed. Vessel regression also occurs in certain tumors which disappear spontaneously. Preliminary observations reveal a progressive narrowing of regressing vessels along their entire length. The vessels become thread-like, devoid of blood flow and disappear. Studies using fluorescein angiography, light and electron microscopy are underway to clarify the ultrastructural details and sequence of events

in vessel regression.

H. Studies of Cartilage Derived Growth Factor and Stimulation of DNA Synthesis in Chondrocytes. A growth factor which stimulates DNA synthesis and cell division in resting monolayers of confluent Balb/c 3T3 cells and bovine chondrocytes has been extracted from bovine scapular cartilage. Chromatography by gel filtration in the presence of 4M guanidine hydrochloride suggests that the growth factor has a molecular weight of approximately 11,000 to 13,000 daltons. Ion exchange chromatography indicates that the growth factor is cationic. The immediate goals are to purify and fully characterize the cartilage derived growth factor, and to make antibodies against it.

II. Studies of the Role of Platelets in the Clearance of Endotoxin

Infants and children with gram negative sepsis develop thrombocytopenia. In a series of laboratory and clinical studies, we have shown that this is because platelets bind endotoxin. In the process of binding endotoxin, platelets also neutralize its activity. We have further shown that rats can be protected from otherwise lethal concentrations of endotoxin injected into the peritoneal cavity, if platelets are also instilled into the peritoneal cavity. The mechanism of this phenomenon is under study.

Division of Plastic Surgery

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Financial Support:
Massachusetts Cosmetology Fund \$3,139, Dr. Murray.

Major Research Projects

I. Growth of Cartilage and Bone

We have employed an experimental model of induced endochondral osteogenesis to study factors important in the maintenance of cartilage and in its removal and replacement by mineralized bone. Subcutaneous implantation of demineralized bone powder into rats triggers a synchronous and highly reproducible sequence of events: attraction of host fibroblasts, followed by the appearance of chondrocytes, and by day nine, calcification of the cartilage matrix occurs coincident with vascular invasion

and chondrolysis. By day fourteen following implantation, the induced plaques demonstrates that the cartilage has been replaced by mineralized bone which will later include hemopoietic marrow. We have altered the temporal sequence of cartilage resorption by inhibition of calcification and blood vessel ingrowth. We hope these studies will provide insights into the mechanisms of abnormalities of skeletal growth and repair and find application in clinical cranio-facial skeletal reconstruction.

II. Biology of Developmental A-V Malformations

Our investigation of the biology of A-V

malformations is directed towards understanding the mechanism of growth and spontaneous involution seen with the common "strawberry" hemangioma. We are examining available surgical specimens by histological, histochemical, and electron microscopic techniques to characterize a lesion's maturity and growth potential. In the future, we hope to utilize explant cultures, autoradiography and study the possible role of "angiogenesis" factors. Through such a study, we hope to develop a clinically useful classification of developmental vascular abnormalities and coordinate a therapeutic program based on biologic criteria.

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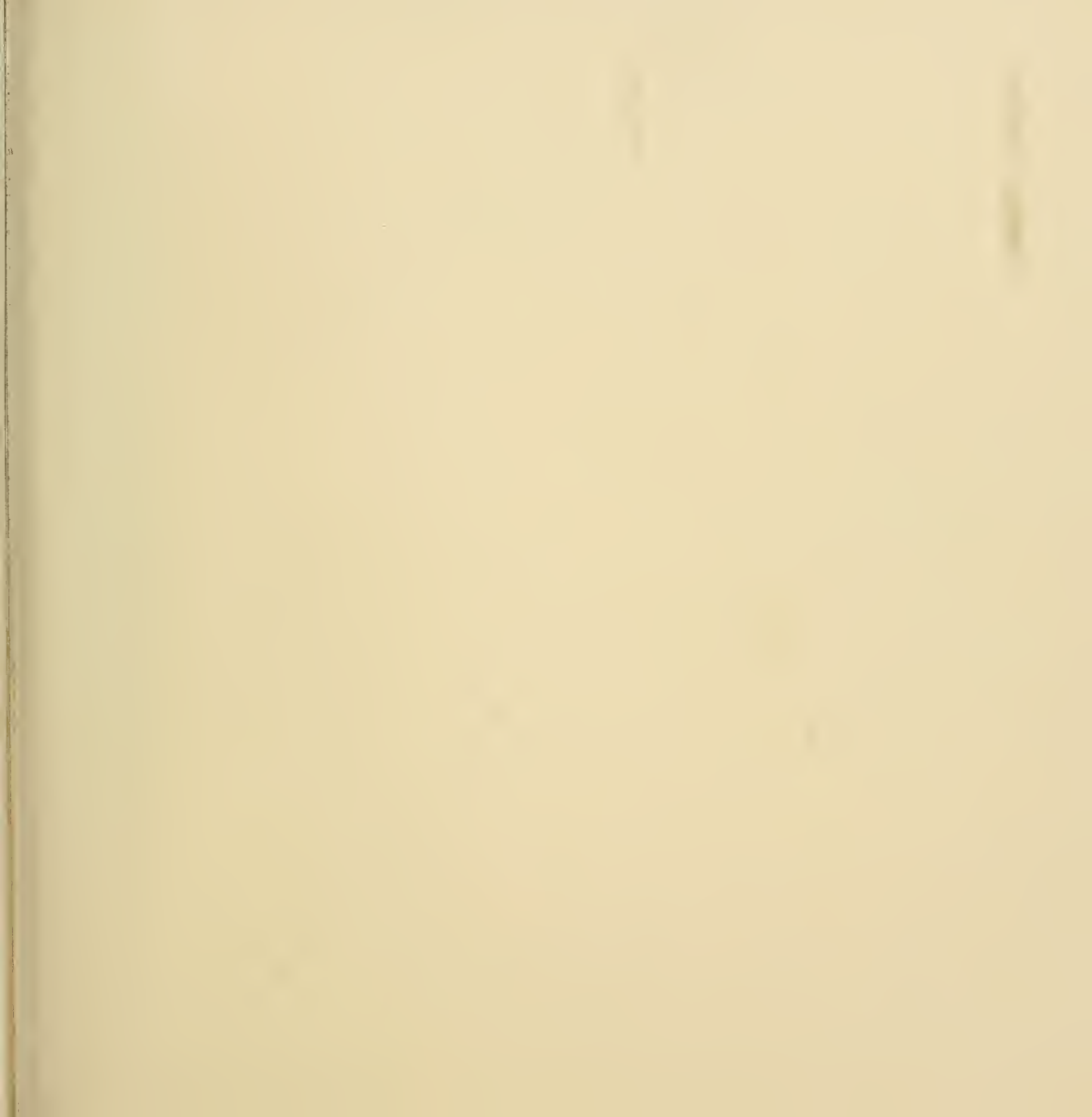
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